

AUTOMATED MICROBIAL
METABOLISM LABORATORY
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Prepared by:

BIOSPHERICS INCORPORATED
4928 Wyaconda Road
Rockville, Maryland 20852

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The participation and assistance of the following Biospherics
personnel is gratefully acknowledged:

Patricia Ann Straat, Ph.D.

Mrs. Margaret Federline

Mrs. Cynthia Nelson

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ABSTRACT

The Automated Microbial Metabolism Laboratory (AMML) 1971-1972 program involved the investigation of three separate life detection schemes. The first was a continued further development of the labeled release experiment. The possibility of chamber reuse without "inbetween" sterilization, to provide comparative biochemical information was tested. Findings show that individual substrates or concentrations of antimetabolites may be sequentially added to a single test chamber. This would allow for relatively extensive biochemical testing with a minimum of engineering changes from the current viking design.

The second detection system which was investigated for possible inclusion in the (AMML) package of assays, was nitrogen fixation as detected by acetylene reduction. The assay appeared to be sample and sensitive and could easily be automated. One gram samples of mud produced a response which was partially light sensitive and four orders of magnitude greater than sterile controls. The possibility of using this assay as a test for photobiology was indicated.

Thirdly, a series of preliminary steps were taken to investigate the feasibility of detecting biopolymers in soil. The most effective extraction of high molecular weight materials was found with methods utilizing sonication and a solvent of 0.2 N NaOH + 0.01 M EDTA. Soil extracts were concentrated by lyophilization and the high molecular weight material obtained by molecular sieve chromatography was determined by optical

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density at 260 mμ. The high molecular weight fraction has been found to contain protein and polysaccharide.

A strategy for the safe return to Earth of a Mars sample prior to manned landings on Mars is outlined. The program assumes that the probability of indigenous life on Mars is unity and then broadly presents the procedures for acquisition and analysis of the Mars sample in a manner to satisfy the scientific community and the public that adequate safeguards are being taken.

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SUMMARY

Labeled Release

Work on the Automated Microbial Metabolism Laboratory (AMML) was conducted to further develop the labeled release experiment. A series of preliminary steps were taken to test the feasibility of using multiple addition test chambers and to lay groundwork for definitive biochemical determinations. Methods for preparing pure culture soil systems were developed and tested.

Experiments were performed to show the relationship between growth and $^{14}\text{CO}_2$ evolution in liquid culture and pure culture soils. In liquid cultures the evolution of radioactivity paralleled the growth of E. coli as measured by turbidity. On the other hand, 0.5 g of soil inoculated with E. coli and charged with 0.1 ml of ^{14}C labeled medium evolved radioactivity, but plate counts failed to show an increase in cell numbers.

Experiments designed to show the feasibility of multiple addition studies showed no difference in the kinetics of $^{14}\text{CO}_2$ evolution using a $\text{Ba}(\text{OH})_2$ collection system when either a 2 ml or 17 ml headspace existed. Carryover effects in multiple addition systems showed little interference from preceding and underlying soil cultures. However, the order of addition of soil and medium did effect the results of a second addition.

A series of ^{14}C labeled substrates was tested on natural, sterile and B. subtilus inoculated Wyaconda soil. They were D-glucose, L-glucose,

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sucrose, lactose, xylose, cellulose, phenylalanine, L-valine, DL-valine, methionine, glycine, L-alanine, D-alanine, citrate and alpha-ketoglutarate.

The natural soil produced positive responses from all substrates tested whereas the B. subtilus amended test soil showed a selective pattern of substrate utilization.

Selected compounds from the same series of substrates were combined to form a carbohydrate medium, an amino acid medium and a Krebs cycle medium. Each of these media was tested with natural Wyaconda soil. In addition, media composed of the individual substrates listed above, were sequentially added along with natural Wyaconda soil, to a single growth chamber (one chamber for each substrate group). No "inbetween" sterilization was done but sufficient time was allowed between additions, for the evolution of radioactivity to reach a plateau.

A comparison between experiments performed in the single addition mode vs. the multiple addition mode showed suprisingly good correlation. The feasibility of using multiple additions to perform a series of individual biochemical tests was demonstrated. However, a carryover effect due to contact of new medium and prior aclimated cells was observed. Further testing should be performed to attempt to minimize or eliminate this effect.

An inhibitor study was conducted in the same fashion as the substrate study, in which various concentrations of iodoacetic acid were mixed with RM9 medium and added to natural Wyaconda soil. In the multiple addition experiment, the order of addition of the inhibitor, i.e., high concentration to low concentration and visa versa, influenced the results.

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When the order of addition was from a high to low concentration, the results most closely approximated results obtained in a single addition mode. As with the substrate study, the multiple addition inhibitor study showed great promise. However, steps need to be taken to minimize the effect of carryover.

Nitrogen Fixation

Preliminary experiments to detect nitrogen fixation in soil by the acetylene reduction method have been performed and the results are highly encouraging. The technique is operationally simple and suitable for automation.

Several soil samples and incubation procedures were investigated and it was found initially that incubation in the light magnified the response from some soils by as much as two orders of magnitude. The use of this assay as a possible monitor for phototrophic activity became obvious.

Incubation in quartz vials in natural light produced the greatest response; however, since temperature, intensity of exposure, etc., were difficult to control, artificial illumination of samples in quartz vials was selected. None of the conditions tested, i.e., quartz, glass, or type of illumination affected the nonbiological response which remained near the level of C_2H_4 contamination in C_2H_2 . One gram samples of Rock Creek Mud showed responses which were as much as four orders of magnitude above sterile controls. Incubation in light produced a 100-fold greater response than incubation in the dark, thus, indicating phototrophic activity.

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Not all samples tested showed acetylene reduction. Soils which had been stored for some time failed to give any response. Freshly collected soils showed a range of responses which was soil dependent and presumably related to the number of nitrogen fixing microorganisms present. No attempt was made to identify and/or enumerate the organisms in soil which were responsible for the response.

Soils were tested under aerobic conditions only. Anaerobic incubation may prove to be a more sensitive determinant of soil activity. However, comparison of the magnitudes of responses under anaerobic and aerobic conditions could provide additional information on the populations present.

A simple experiment to determine the time course of acetylene reduction by Azotobacter vinelandii was performed. Near maximum response occurred after 50 hours. The heat sterilized control showed an initial activity which might be caused by heat stable enzymes. Further studies obviously need to be performed. However, this initial work indicates; the ease with which the technique is performed, the high level of sensitivity, the lack of interference, the ability to detect photobiology, and the low background.

Biopolymers

A pilot study to determine the feasibility of detecting high molecular weight polymers (10,000) in soil has been performed. The following results have been demonstrated by these studies:

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1. Soil extracts contain large amounts of material which reacts with the Folin protein reagent. Protein is found in the high molecular weight fractions. However, proteins represent only a small percent (1 - 10%) of the total Folin-reacting material. The remainder consists of low molecular weight compounds.
2. Polysaccharide material is also found in the high molecular weight fractions.
3. Sonication provides the highest yields of Folin-reacting material. Grinding of the soil is most effective as an extraction technique when the soil/solvent mixture forms an abrasive paste. However, grinding has never been more than 75% as effective as sonication.
4. The most successful solvent used for extraction has been 0.2 N NaOH + 0.01 M EDTA which provided yields of 2 μ g protein/g soil. Milder extraction methods (0.1 M Tris buffer pH 7.0) were not as successful and provided yields of only 0.2 μ g protein/g soil.
5. Considerable variability in yield was seen on a daily basis. Protein yields were higher from fresh soil than from soil stored for one day.
6. Soil extracts are best concentrated by lyophilization.
7. Lyophilized soil extracts may be dissolved or suspended in aqueous solution and placed on molecular sieve columns.

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- a. The presence of high molecular weight material has been detected by observing the optical density of the eluted material at 260 mμ.
 - b. The high molecular weight fraction has been shown to contain protein and polysaccharide material.
 - c. Bio-Gel P-10 is superior to Sephadex G-25 in resolution of high from intermediate molecular weight material.
8. As determined by separation properties, molecular sieve resins are stable to dry heat treatment for one hour at 125°C. Since Sephadex G-25 may undergo small pH changes during this heat treatment, Bio-Gel P-10 is the preferred resin.

In summary of these experiments, we have demonstrated in a pilot study, the feasibility of extracting biopolymers from soil which are at least 10,000 in molecular weight. These biopolymers can be concentrated by lyophilization to enable their subsequent detection after separation from smaller compounds by molecular sieve chromatography. The methodology described is readily adaptable to automated planetary landers because extraction can be performed by grinding, molecular sieve resins can be sterilized by dry heat treatment, and suitable column technology has already been developed. An important additional advantage is that the biopolymeric fraction separated by chromatography is the first fraction

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eluted from the column. We conclude that biopolymer detection in soil according to the described techniques is feasible and provides a new non-geocentric life detection scheme for planetary probes.

Prior to risking manned landings on Mars, a program of examining samples of Mars soil returned to Earth by automated landers should be carried out to determine if Mars soil imposes any hazard to astronauts or to terrestrial life. It is proposed that a representative variety of discrete samples be obtained from Mars for examination on Earth or, preliminarily, on an intermediate station on the Moon or aboard an Earth orbiting satellite. The Mars environment should be maintained during the return flight. The containers should be designed to permit key examinations to be conducted without opening the seal. The experiments should be conducted behind a biological barrier as should the subsequent analyses after the sample containers are opened. Depending on the test results, decisions on further distribution and examination of the samples should be made.

A table is presented which presents the types of tests recommended for determining the hazards posed by the Mars samples. The use of unicellular organisms as potential hosts is emphasized in the bioassay portion of the testing. The tests should incorporate, to the extent feasible, existing NASA life detection instrumentation and, in any event, must be remotely performed behind the biological barrier. For the purposes of this important

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aspect of the NASA planetary exploration program, it is recommended that the probability of indigenous life on Mars be taken as unity.

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I. LABELED RELEASE

A. Introduction

Of all experiments which are included in the AMML package, the labeled release is the most sensitive and versatile from the standpoint of pathway differentiation. An almost unlimited array of ^{14}C labeled organics may be utilized to establish a profile of specific metabolic capabilities for organisms contained in a soil sample. In addition, an established medium may be used under an array of test environmental conditions to determine growth or metabolism as affected by those conditions. Some of the conditions which may be imposed are the control of temperature, light, water and the addition of inhibitors, including various gases. In fact, the possibilities for different specific experiments to establish metabolic characteristics are almost limitless. While several "most likely" candidates may be suggested on the basis of previous experience and current work, a comprehensive theoretical and experimental examination of possibilities should be made in achieving a selection. The present approach is to develop methodology to allow successive experiments to be conducted in a single chamber. This technology could then be applied in comprehensive experiments designed to perform a sequence of individual experiments.

In order to demonstrate the feasibility of establishing a metabolic profile which characterizes the unknown organism, pure culture test

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organisms must be utilized. Natural soils contain mixed populations of microorganisms which represent a spectrum of trophic levels, environmental tolerances and metabolic activities. Each organism species occupies a specific niche in the ecology of soil such that few potential sources of energy or carbon go untapped once they percolate into the soil. For this reason, natural soil displays a rather nonselective response to a broad array of ^{14}C labeled naturally occurring organic substrates. However, individual species of the population, when tested separately, do display very selective and sometimes characteristic biochemical and physiological capabilities. It is this highly evolved selectivity which maintains the integrity of the species.

If Martian soils contain mixed populations of organisms with metabolisms similar to that of organisms on Earth, then a relatively nonspecific response from an array of substrates and environmental conditions would be expected. On the other hand, if Martian organisms differ in biochemical capabilities, then a selective pattern of substrate utilization would occur.

Preliminary testing of this approach was performed in a series of steps. Initially it was necessary to prepare pure culture soil systems which would simulate the natural soil. These inoculated soils were tested for bacterial growth and $^{14}\text{CO}_2$ evolution in comparison with the liquid culture system. A prerequisite to performing sequential addition

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experiments was the determination of possible carryover interference and the effects of a changing head space. Several experiments designed specifically to test these effects were conducted. In addition, a series of ^{14}C labeled substrates were individually tested. These were to provide a comparison for results of sequential addition systems and to demonstrate the selectivity of a pure culture soil as opposed to natural mixed population soils.

B. Multiple Addition Preliminary Studies

As a preliminary to feasibility testing of the multiple addition mode of comparative biochemical tests, a series of experiments were necessary. These included preparation and testing of pure culture soils, the relationship of CO_2 evolution to growth in soils, the effect of a varying headspace volume and the carryover effect of sequential additions.

Two methods of preparing pure culture tests soils were:

- 1.) inoculation of heat-sterilized soil followed by drying and screening,
- 2.) inoculation of heat-sterilized soil just prior to addition of ^{14}C labeled medium.

The former method was used with B. subtilus since only hardy and/or spore-forming organisms would survive drying and subsequent storage. Although this technique is limited to few organism species, the resulting soil may be handled in similar fashion to natural viable soil. The prepared soil is stable and a series of experiments may be conducted using inoculated soil from a single batch preparation.

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The inoculation of soil just prior to testing may be used with a greater variety of organisms since long-term survival in the soil is not necessary. However, it appeared that survival of some cells may be better in soil than without it. Figure 1 shows that the initial evolution of radioactivity by Pseudomonas aeruginosa was greater when only ^{14}C medium and cells were mixed than when ^{14}C medium, cells and soil were mixed. However, after approximately 24 hours of incubation, the system with soil began a rapid evolution of radioactivity not seen after 56 hours in the system without soil. This likely indicates that the P. aeruginosa adapted to the soil environment and was able to metabolize and, perhaps, grow whereas the medium without soil failed to support this level of activity. This type test system would, therefore, be suitable for pure culture studies.

Soils inoculated with Bacillus subtilis were prepared in the following way: A quantity of Wyaconda soil was weighed and sterilized by heat. A suspension of B. subtilis in H_2O was then added to the sterile soil and stirred to make a homogeneous slurry. Afterwards the slurry was allowed to air dry under sterile conditions and was sieved and stored in sterile bottles. The calculated cell density, based upon microscopic cell count of the initial pure cell suspension, was 5×10^5 cells per gram of soil. An experiment using 0.5 g of this inoculated soil, performed approximately one month after preparation of the soil, is shown in

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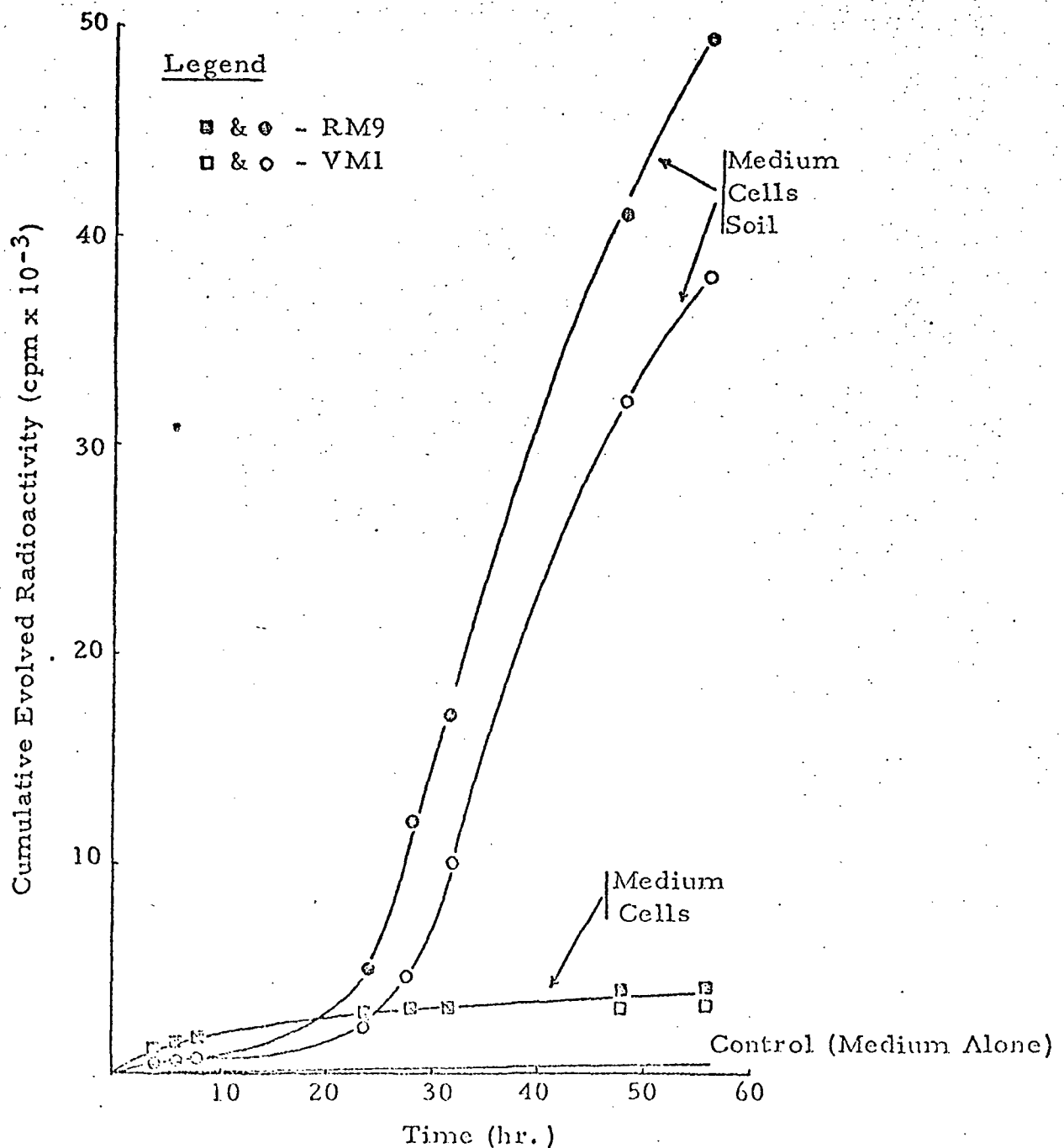
FIGURE 1

Evolution of $^{14}\text{CO}_2$ by *Pseudomonas aeruginosa*

Soil: 1 g heat sterilized Wyaconda soil

Medium: 0.1 ml modified RM9 or VM1 without glycolate ($15 \mu\text{Ci/ml}$)

Inoculum: 0.1 ml of *P. aeruginosa* 5×10^6 cells/ml in normal saline, added just prior to medium.



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Figure 2. In this experiment the medium consisted of only UL ^{14}C -D-glucose ($10\ \mu\text{Ci/ml}$, 10^{-3}M). This inoculated soil provides a pure culture system which most closely approximates a natural dry soil having relatively low numbers of cells.

Experiments which are shown in Figures 3 and 4 were conducted to determine the relationship of $^{14}\text{CO}_2$ evolution and growth in the moist inoculated soil as compared with liquid cultures. In the liquid culture, E. coli evolved radioactivity and this evolution was paralleled by an increase in optical density. Clearly the metabolism of ^{14}C labeled substrates was accompanied by growth of the organism. On the other hand, ^{14}C labeled medium added to soil and E. coli resulted in the evolution of radioactivity, but not an increase in cells.

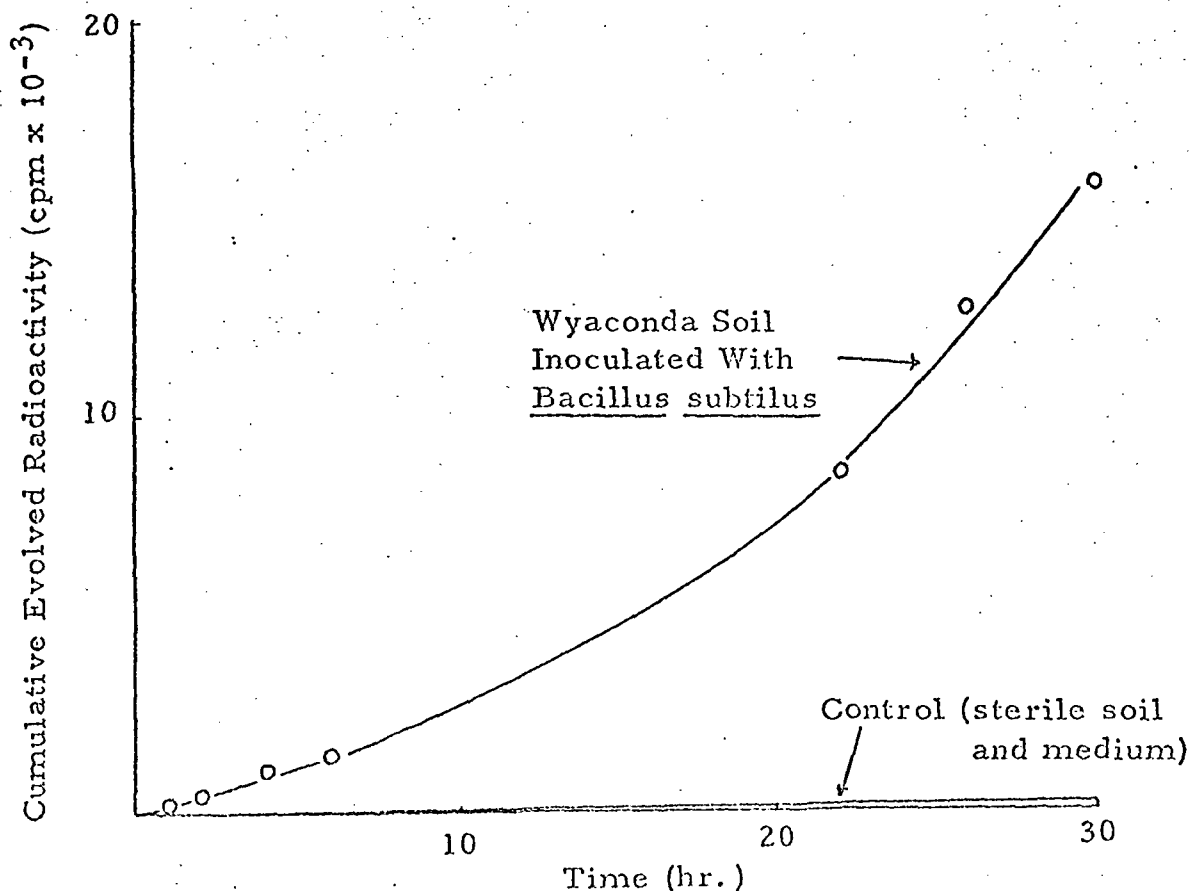
In view of the fact that multiple additions of soil and medium would result in a changing headspace volume, it was deemed necessary to determine if this change would alter the rate or efficiency of $^{14}\text{CO}_2$ production and collection with $\text{Ba}(\text{OH})_2$. Two test culture systems, as shown in Figure 5, were compared. One-half gram portions of Wyaconda soil were placed in the 17 ml vials and 2 ml planchets. Control soils from each were sterilized by dry heat (30 min., 212°C) prior to medium addition. VM1 medium (without glycolic acid, total radioactivity $15\ \mu\text{Ci/ml}$) was added to each system and the evolved $^{14}\text{CO}_2$ was collected. Results of viable soil in the 17 ml vial and 2 ml planchet were very similar (Table 1),

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FIGURE 2
Evolution of $^{14}\text{CO}_2$ by Bacillus subtilis

Soil: 0.5 g of Wyaconda soil which was inoculated with 2.5×10^5 cells of B. subtilis dried and stored one month prior to use.

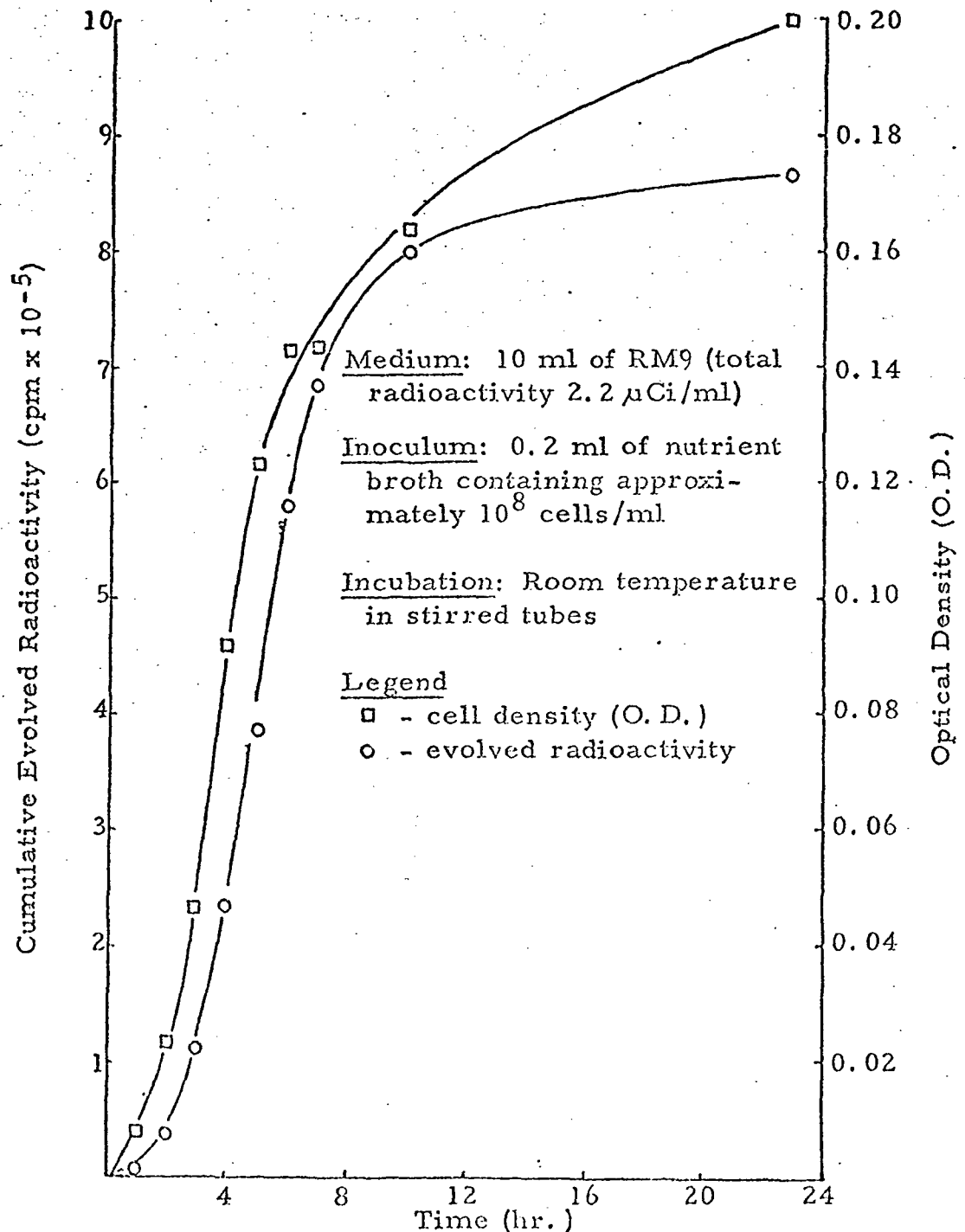
Medium: 0.1 ml of distilled H_2O containing UL ^{14}C D-glucose (1 mM, $10 \mu\text{Ci/ml}$).



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FIGURE 3

Evolution of $^{14}\text{CO}_2$ and Optical Density
of a Liquid Culture of E. coli



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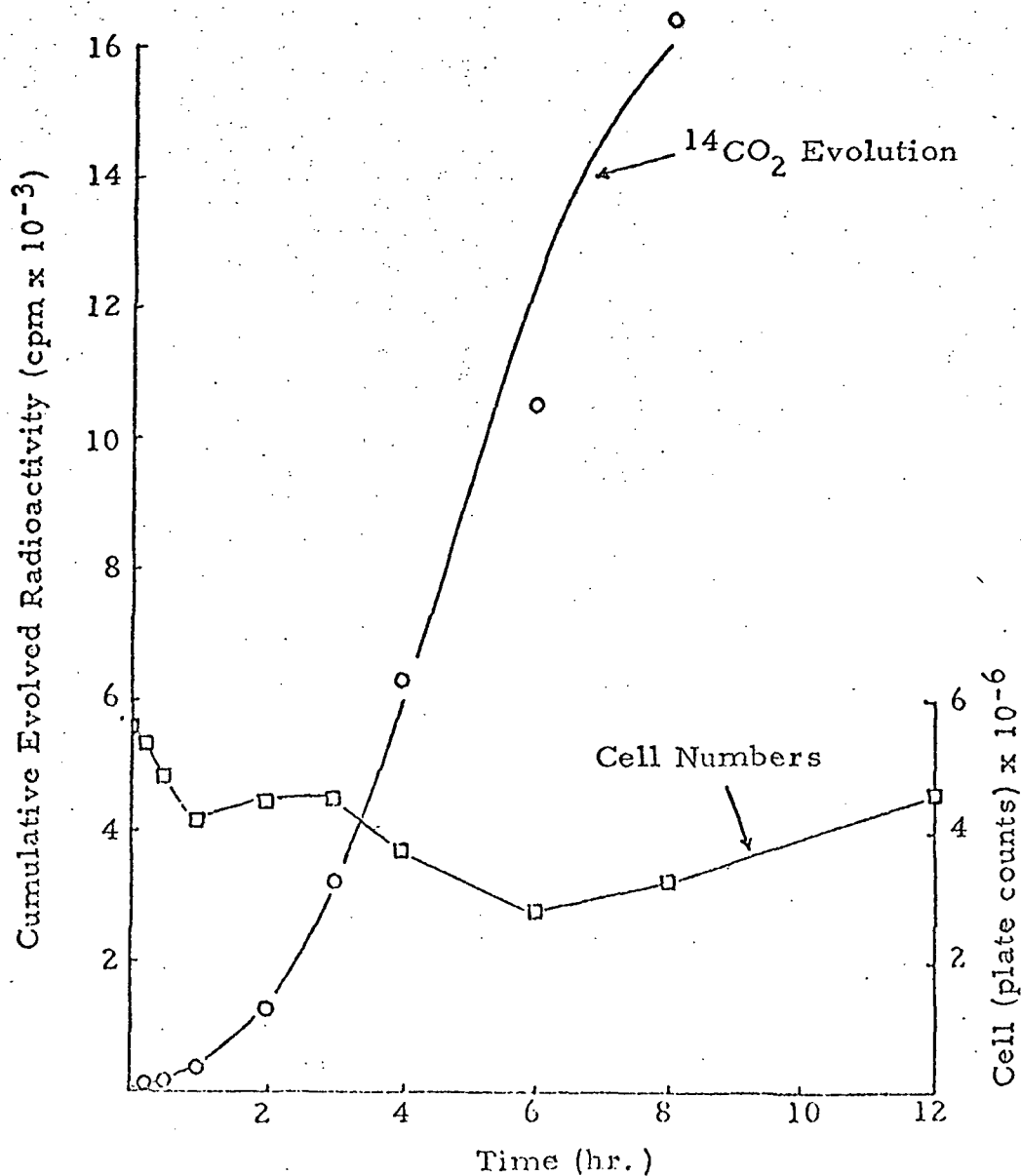
FIGURE 4

Evolution of $^{14}\text{CO}_2$ and Cell Density of A Moist Culture of E. coli

Medium: 0.1 ml of VM1 without glycolate (total radioactivity 15 uCi/ml)

Inoculum: 0.1 ml of E. coli 6×10^7 cells/ml.

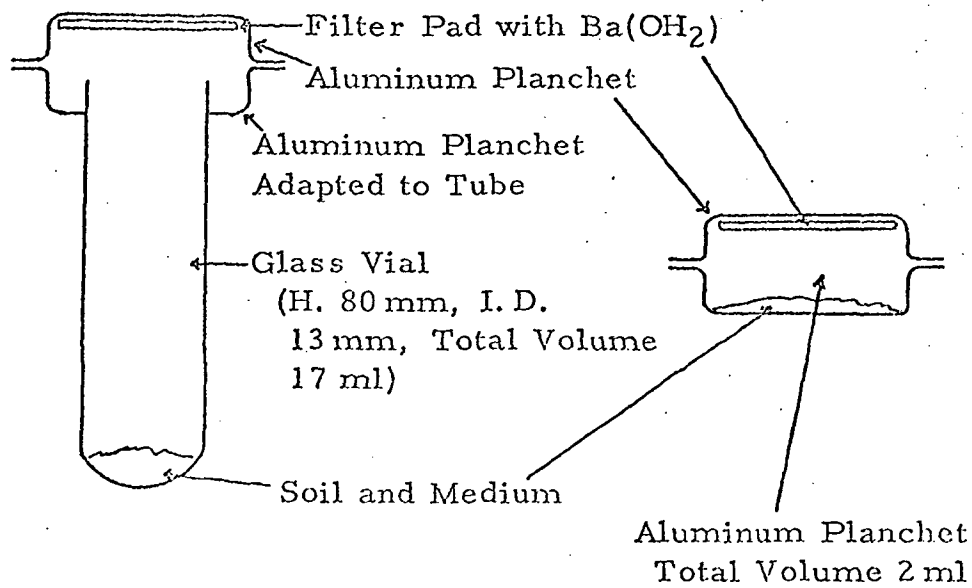
Incubation: Room temperature in stirred tubes.



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FIGURE 5

Culture Systems Which Were Compared
to Determine the Effect of Head Space



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TABLE 1

Labeled Release Conducted in 2 ml Planchet and 17 ml Vial

Time (hr.)	Cumulative Evolved Radioactivity (cpm)							
	2 ml Planchet				17 ml Vial			
	Viable		Sterile		Viable		Sterile	
1	474	460	96	73	597	413	41	33
3	1977	2057	257	298	2210	1683	96	102
5	3639	3884	463	511	4347	3197	174	155
8	8997	7490	852	916	8371	7088	168	235

Soil: 1g Wyaconda

Medium: 0.1 ml VML without glycolate total radioactivity 15 uCi/ml

Incubation: room temperature

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thus indicating that the diffusion time and efficiency of the $^{14}\text{CO}_2$ collection were not significantly different in the two systems.

The possible influence of earlier and underlying soil and medium on the response obtained from subsequent additions was tested. It had been shown in earlier experiments (Figure 6) that the addition of ^{14}C labeled medium and soil to a ^{14}C labeled medium soil system which had reached a plateau produced an immediate and rapid evolution of radioactivity.

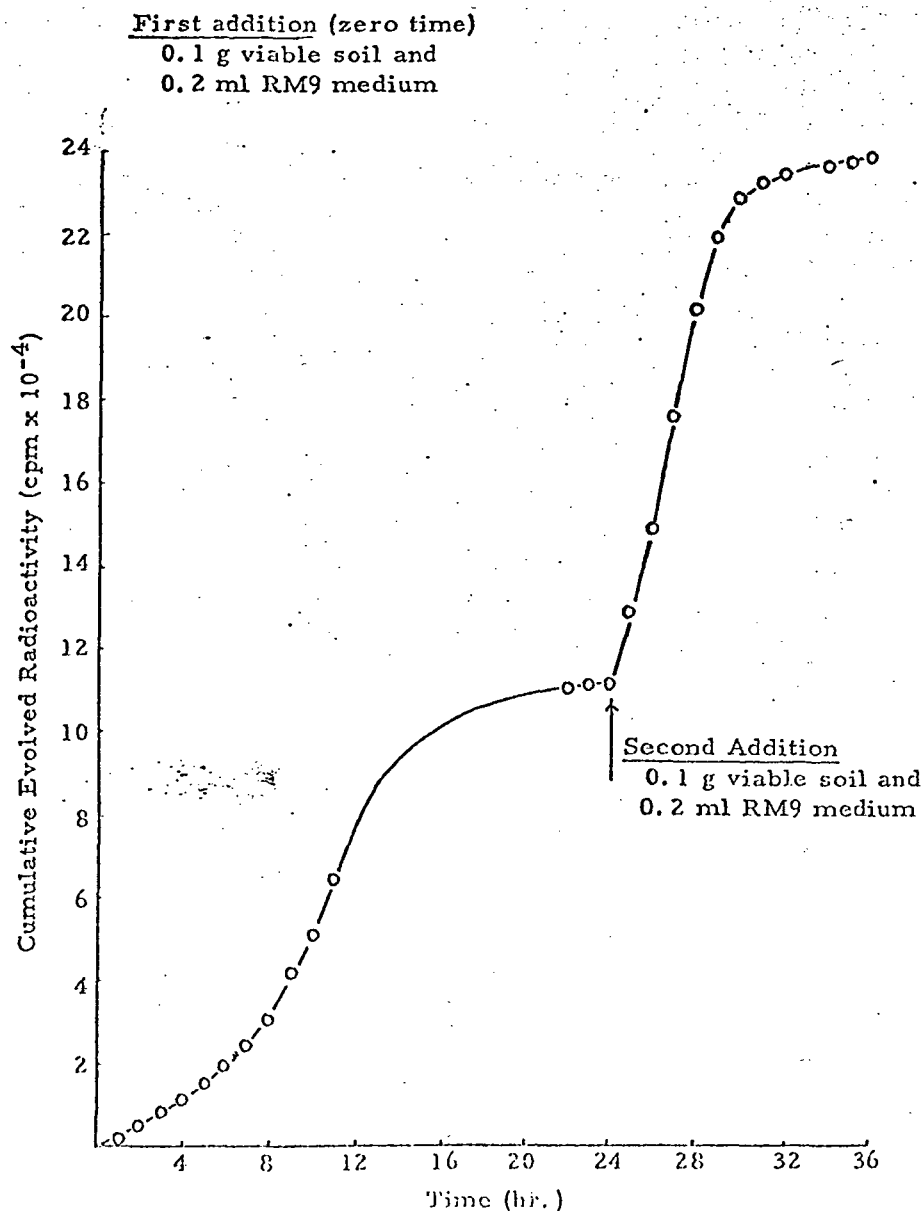
Since a large percent of the metabolized ^{14}C organic material is incorporated into cell material and not evolved as $^{14}\text{CO}_2$, the possibility existed that cells might evolve this fixed ^{14}C at a later time, especially as the result of renewed activity which occurs after a second dose of a usable substrate. Such a latent evolution of radioactivity could confuse the results of a subsequent addition. To test the possible stimulation of a latent release, soils were dosed with ^{14}C labeled VM1 medium and the evolved $^{14}\text{CO}_2$ collected until a plateau in evolution had occurred. A second addition of soil and VM1 (unlabeled) or soil and H_2O was then made. As shown in Figure 7, no significant evolution of radioactivity occurred.

A second experiment was conducted in which the order of addition of soil and medium were varied. This experiment is shown in Figure 8. There was no difference in the response obtained during the first addition

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FIGURE 6

Multiple Addition of Viable Soil and Labeled Medium *



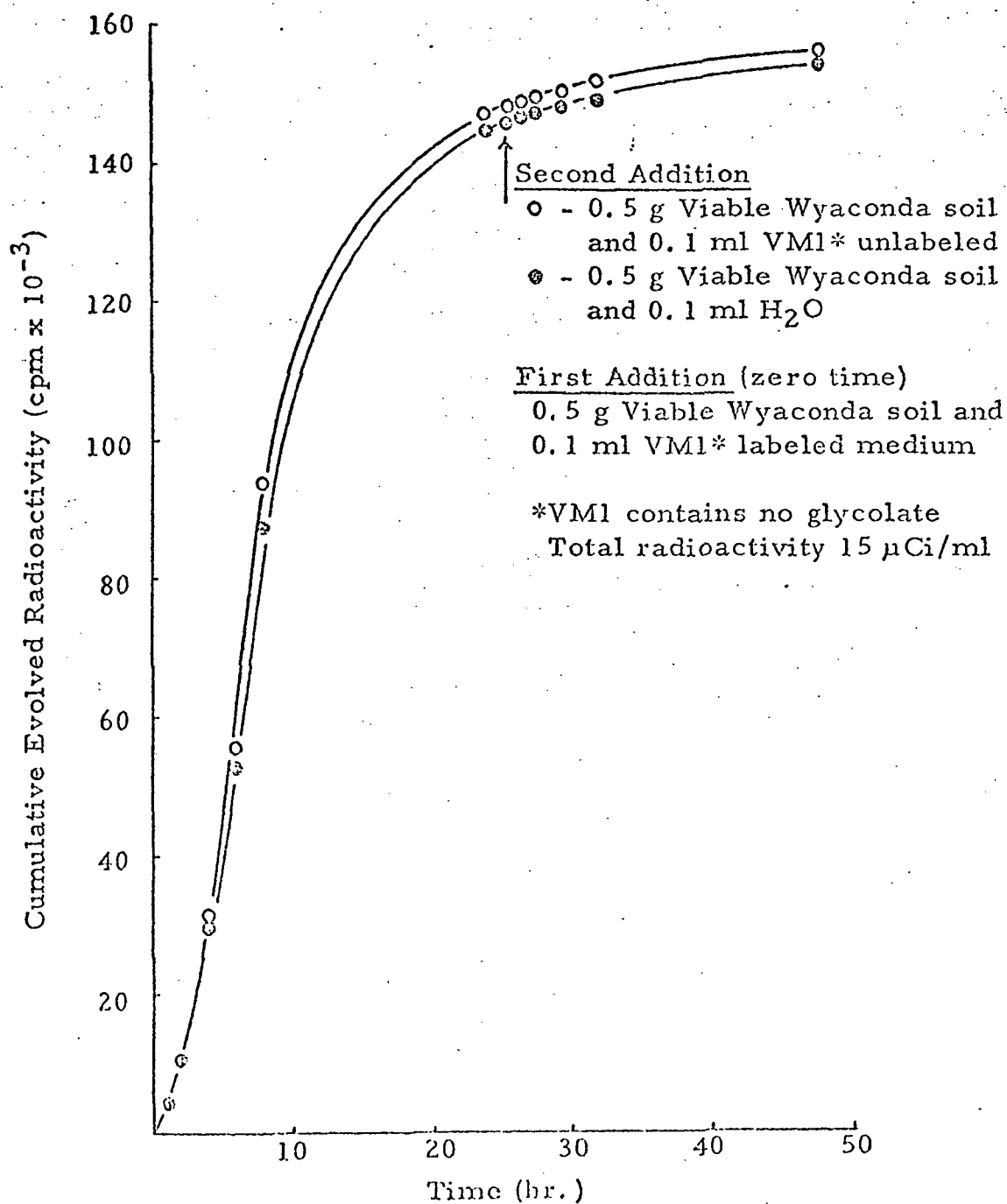
Taken From:

- * Participation in the Science Planning for the Viking 1975 Missions in the Area of Biology. First Monthly Progress Report, NAS1-9690
10 July 1970

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FIGURE 7

Effect of Adding H_2O or Unlabeled Medium
to a Soil Culture Which has Reached a Plateau
in the Evolution of $^{14}CO_2$ from Labeled Medium



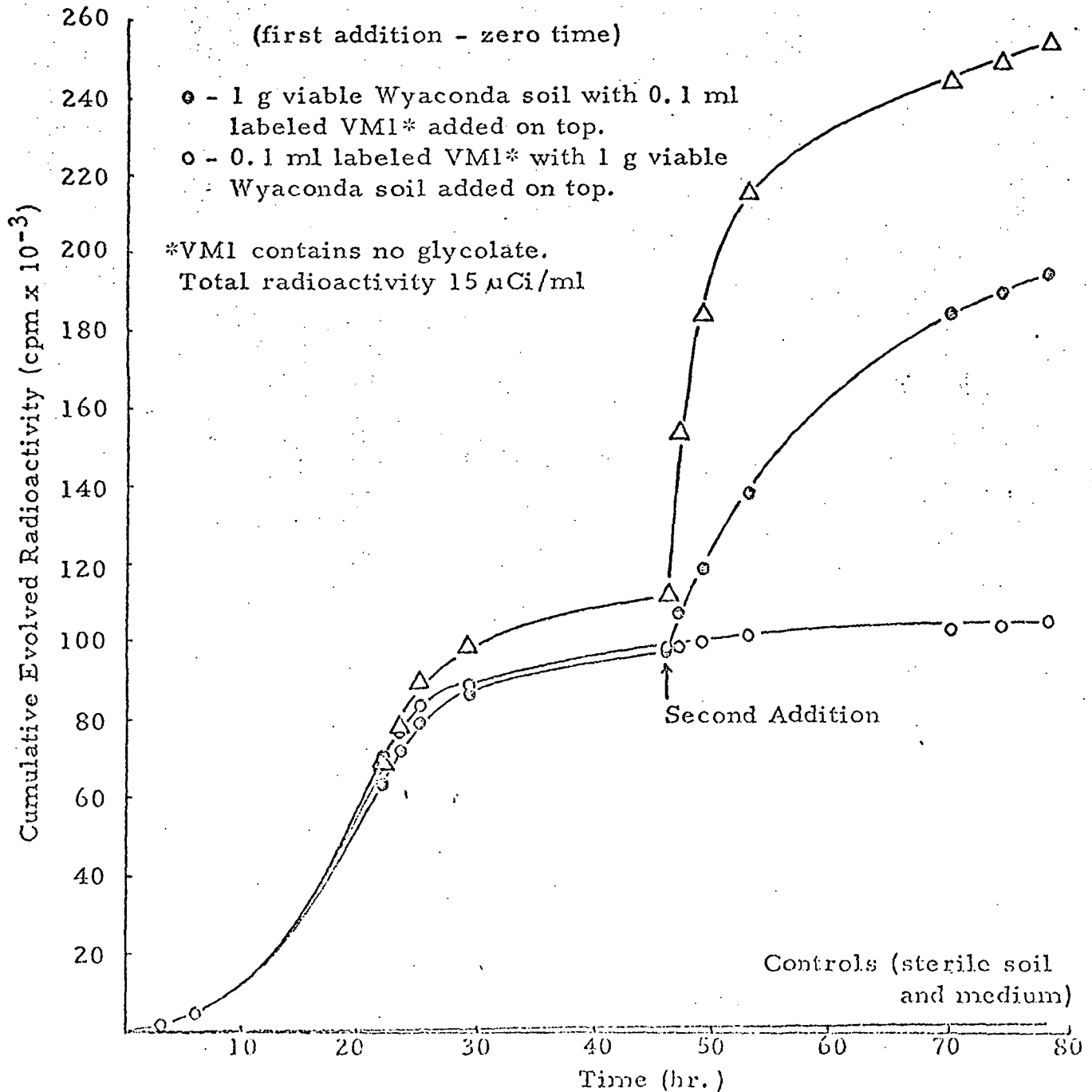
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FIGURE 8

Effect of the Order of Addition of Medium and Soil

Legend (second addition)

- Δ - 0.1 ml labeled VM1 with 3 g of viable Wyaconda soil added on top.
- \bullet - 1 g viable Wyaconda soil with 0.1 ml labeled VM1 added on top.
- \circ - 1 g viable Wyaconda soil with 0.1 ml nonlabeled VM1 added.



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when 0.1 ml of VM1 medium was added to the top of a 1.0 g sample of soil or when the additions were made in the inverse order. It appears that metabolically produced $^{14}\text{CO}_2$ passes quickly up through the overlaying soil with no significant effect on gas collection.

The response obtained after the second addition of soil and medium was influenced by the order of addition. Labeled medium placed directly on the previously charged soil then overlayed with soil, resulted in a rapid evolution of radioactivity. But when the second addition was made by placing fresh soil on the previously charged soil and dosing this with ^{14}C labeled medium, the response was much slower. This latter technique produced a cumulative response which approximated that achieved during the first addition. Making the second addition, medium then soil, resulted in a much greater cumulative evolution. The relatively small quantity of medium (0.1 ml) resulted in only a partial wetting of the 1.0 g of soil. Since the second addition of soil topped by medium produced a non-lag response, indicating that it came from previously conditioned organisms, it must be assumed that some of the medium percolated down to the level of contact between the new and old soil. Enough previously acclimated organisms were reached to elicit the non-lag response seen; however, most of the contact between organisms and medium appeared to be in the newly added soil. This was not the case when medium was added directly to the previously charged soil. Organisms there were well acclimated to the

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medium and a more rapid and greater quantitative evolution of radioactivity occurred.

Also shown in Figure 8, is that a second addition of soil and non-labeled medium does not result in the evolution of previously incorporated ^{14}C substrates, even though a metabolism as seen when labeled medium is added must be assumed.

Completion of this series of preliminary experiments supported the feasibility of an approach, whereby successive specific tests of substrates and inhibitors could be tested in a single growth chamber without "in between" sterilization of the chamber. It appeared that underlying and completed test soil did not seriously affect later experiments, changes in headspace and chamber configuration due to soil addition did not affect $^{14}\text{CO}_2$ evolution and collection, and the preparation of pure culture soils was possible for simulated comparative biochemical studies.

C. Substrate Specificity

Experiments were conducted in which a series of ^{14}C labeled substrates were tested on natural soil, sterile soil inoculated with Bacillus subtilus, and sterile soil. A list of substrates which were tested is shown in Table 2. In addition to the substrates shown in that list, two fatty acids were also tested. These were 1 ^{14}C N-butyric acid and 10 ^{14}C oleic acid. Problems of solubility and high background rendered these substrates unsuitable. All substrates were individually placed on the above soils as

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TABLE 2

Substrates Which Were Used in Specificity Study

Substrate	Concentration	Added Radioactivity	Measured Radioactivity†	Volume of Medium
1 ¹⁴ C D-glucose	1.0 x 10 ⁻³ M	10 μCi/ml	98,000 (cpm)	0.1 ml
1 ¹⁴ C L-glucose	1.0 x 10 ⁻³ M	10 μCi/ml	98,000 (cpm)	0.1 ml
UL ¹⁴ C sucrose	1.0 x 10 ⁻³ M	10 μCi/ml	125,000 (cpm)	0.1 ml
1 ¹⁴ C lactose	1.0 x 10 ⁻³ M	10 μCi/ml	101,000 (cpm)	0.1 ml
UL ¹⁴ C D-xylose	3.3 x 10 ⁻³ M	10 μCi/ml	104,000 (cpm)	0.1 ml
UL ¹⁴ C cellulose*	1.8 mg (dry)	10 μCi/1.8 mg		0.1 ml (H ₂ O)
Ring 1 ¹⁴ C				
DL phenylalanine	4.7 x 10 ⁻³ M	10 μCi/ml	104,000 (cpm)	0.1 ml
1 ¹⁴ C L-valine	1.0 x 10 ⁻³ M	10 μCi/ml	85,000 (cpm)	0.1 ml
1 ¹⁴ C DL-valine	1.0 x 10 ⁻³ M	10 μCi/ml	89,000 (cpm)	0.1 ml
1 ¹⁴ C DL-methionine	1.0 x 10 ⁻³ M	10 μCi/ml	97,000 (cpm)	0.1 ml
UL ¹⁴ C glycine	1.0 x 10 ⁻³ M	10 μCi/ml	107,000 (cpm)	0.1 ml
UL ¹⁴ C D-alanine	1.0 x 10 ⁻³ M	10 μCi/ml	99,000 (cpm)	0.1 ml
UL ¹⁴ C L-alanine	1.0 x 10 ⁻³ M	10 μCi/ml	103,000 (cpm)	0.1 ml
1 ¹⁴ C citric acid	1.0 x 10 ⁻³ M	10 μCi/ml	202,000 (cpm)	0.1 ml
UL ¹⁴ C α-keto-glutaric acid	1 x 10 ⁻³ M	10 μCi/ml	113,000 (cpm)	0.1 ml

†(0.02 ml medium + 0.2 ml Ba(OH)₂ + 0.4 ml H₂) dried under infra-red lamp in concentric stainless steel planches and counted.

*Since the cellulose was not H₂O soluble, a 1.8 mg portion was sprinkled onto the test soil and 0.1 ml of sterile H₂O added.

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single addition experiments, as well as multiple additions. Also, media containing several substrates which belonged to a single class of compounds were prepared and tested, i. e., carbohydrates, amino acids, Krebs cycle intermediates.

1. Single Addition

The single addition experiments were all conducted in aluminum planchets. (soil 0.5 g) was weighed into the planchets and medium (0.1 ml) was added. Incubation was at room temperature and evolved $^{14}\text{CO}_2$ was collected with $\text{Ba}(\text{OH})_2$. Sterile controls were heat treated (212°C , 30 minutes). All experiments were conducted in duplicate and the results shown are the average of these duplicates.

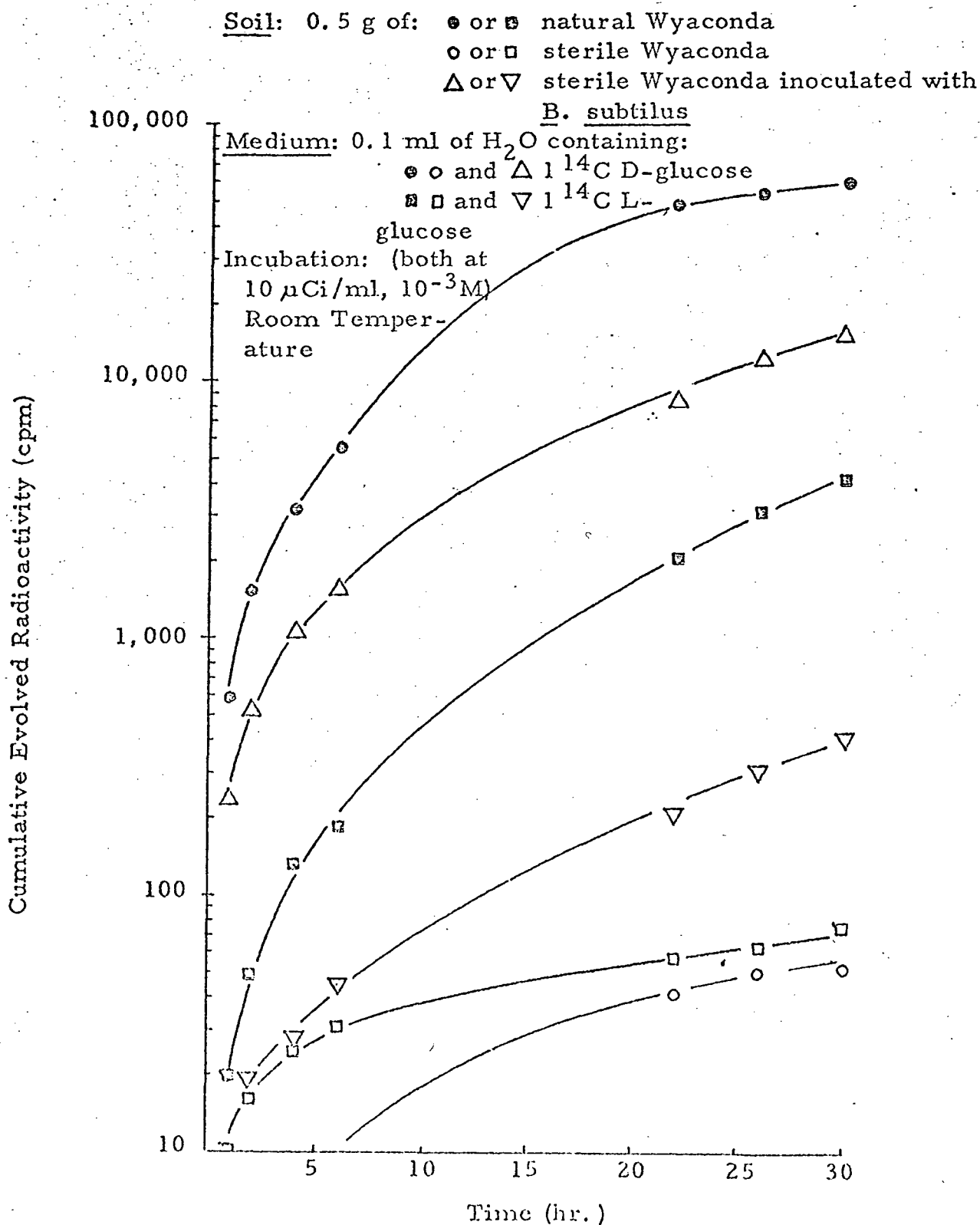
$1\ ^{14}\text{C}$ D-glucose (Figure 9)
 $1\ ^{14}\text{C}$ L-glucose (Figures 9 and 10)

$1\ ^{14}\text{C}$ D-glucose produced the greatest viable to sterile response ratio of the 15 substrates tested. The sterile control was low, and both the inoculated and untreated soils produced rapid rates of evolution. Unexpected results were obtained with L-glucose. It was thought that a biological response would not be obtained with L-glucose since the biological metabolism of this compound has not been previously reported. The cumulative response was not as great as that from D-glucose (100,000 cpm in 160 hours); however, the viable to sterile ratio was 44 fold, and an extended incubation (see Figure 10) resulted in a

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FIGURE 9

Evolution of $^{14}\text{CO}_2$ from 1 ^{14}C D-Glucose and 1 ^{14}C L-Glucose



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FIGURE 10

Evolution of $^{14}\text{CO}_2$ from 1 ^{14}C L-Glucose During Extended Incubation

Soil: 0.5 g of \bullet \circ & \blacksquare natural Wyaconda
 \triangle & \square sterile Wyaconda

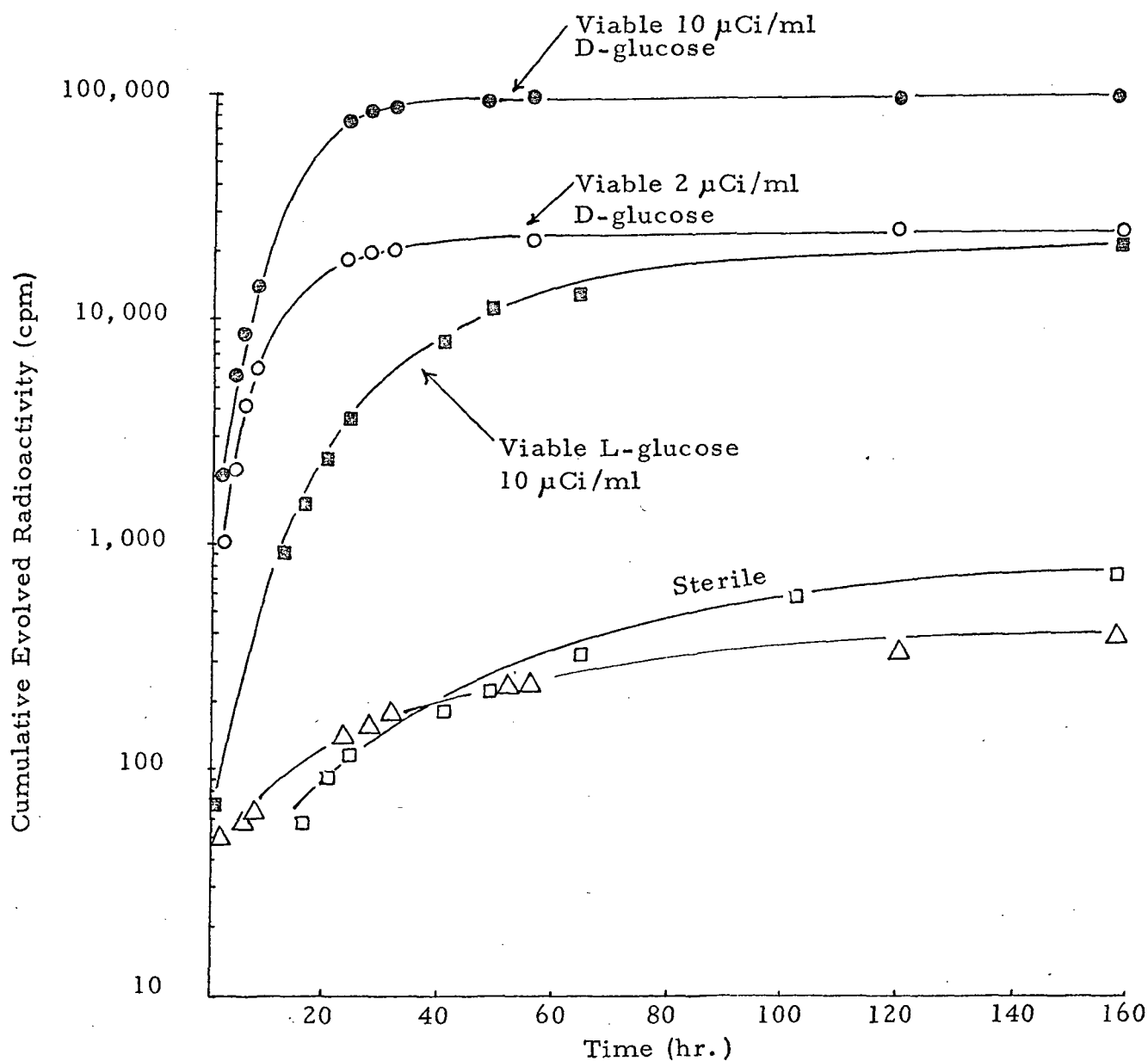
Medium: 0.1 ml of H_2O containing:

\bullet and \triangle 1 ^{14}C D-glucose, $10\ \mu\text{Ci/ml}$, 10^{-3}M

\blacksquare and \square 1 ^{14}C L-glucose, $10\ \mu\text{Ci/ml}$, 10^{-3}M

\circ 1 ^{14}C D-glucose, $2\ \mu\text{Ci/ml}$, $2 \times 10^{-4}\text{M}$

Incubation: Room Temperature



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a cumulative evolution of 20,000 cpm after 160 hours. A comparison of the L and D responses would mean that the material purchased as ^{14}C L-glucose would have had to contain approximately 20% impurities if the L-glucose were not metabolized. The supplier (New England Nuclear) was contacted and questioned concerning the purity of the material. They insisted that the ^{14}C L-glucose was radiochemically 99% pure as determined by thin layer chromatography.

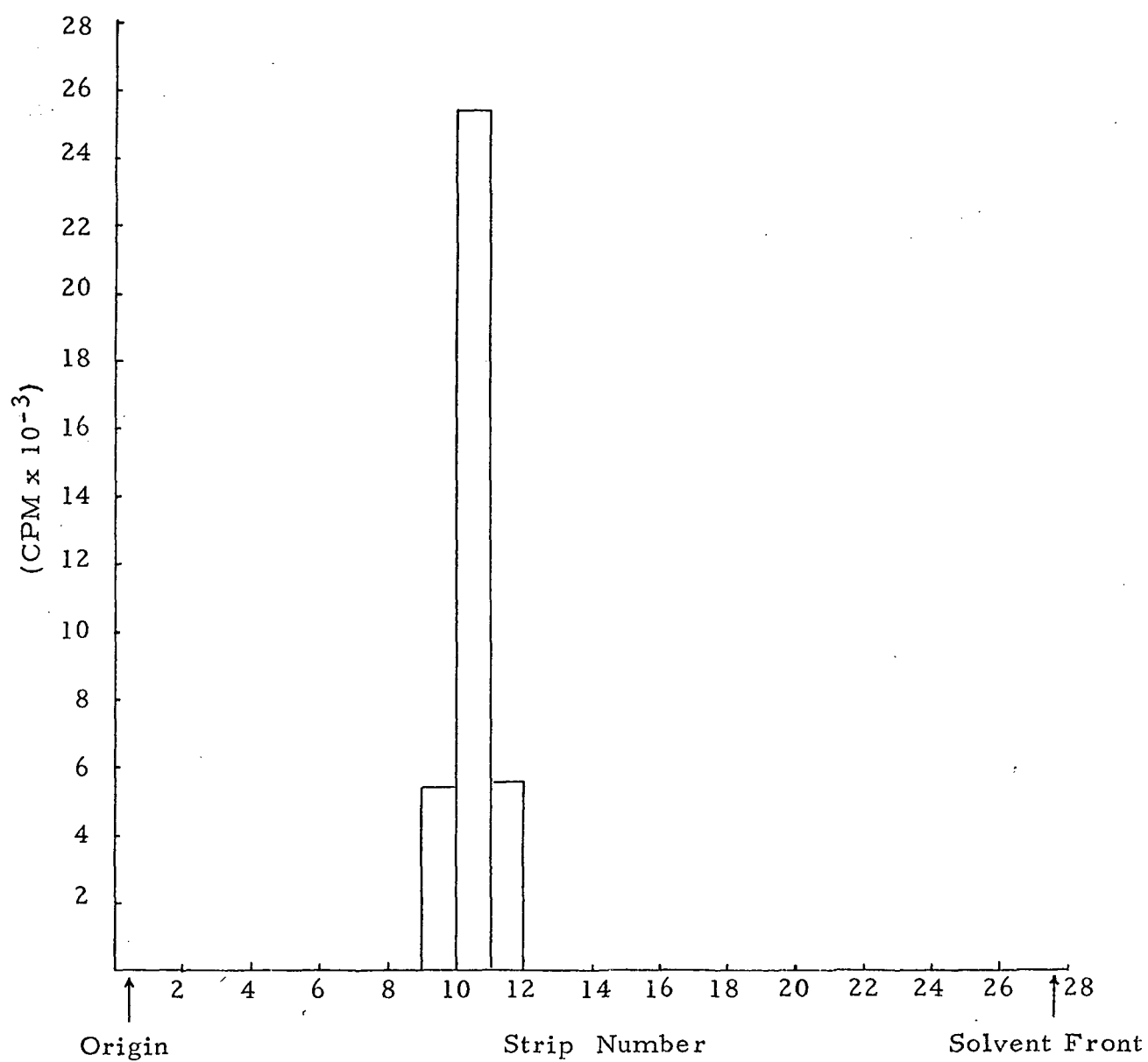
Thin layer chromatography was also performed by Biospherics. Samples of the ^{14}C L-glucose (0.02 μCi and 0.04 μCi) were spotted along with unlabeled L-glucose (5.63 μg). An n-Butanol:acetone: H_2O (30:50:20) solvent system and development with aniline phthalate spray was used. Only one spot having an R_f of (0.39) which corresponded to the R_f for L-glucose (0.38) was observed. The chromatogram was cut into strips and each strip cut into 28 five millimeter wide sections. Each section was placed into a liquid scintillation vial containing 2, 5 - Diphenyloxazole (16.5 g), 1, 4 - bis - 2 (5-phenyloxazolyl) benzene (0.5 g), Toluene (1.3 liters) and Triton X (0.67 liters).

The distribution of radioactivity on the chromatogram was as shown in Figure 11. Ninety-seven percent of all radioactivity on the chromatogram was found in the single peak. The ^{14}C glucose appeared to be free of other ^{14}C labeled organics. The possibility exists, however, that the ^{14}C glucose (listed as ^{14}C L-glucose by the manufacturer) actually contained

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FIGURE 11

Distribution of Radioactivity
on Chromatogram of ^{14}C L-Glucose



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substantial quantities of ^{14}C D-glucose. Although specific proof for the absence of ^{14}C D-glucose in the ^{14}C L-glucose is lacking, the curve for $2\text{ }\mu\text{Ci/ml}$, $2 \times 10^{-4}\text{ M}$ ^{14}C D-glucose, also shown in Figure 10, provides evidence that the metabolized substrate in the ^{14}C L-glucose is not ^{14}C D-glucose.

The reduced concentration of ^{14}C D-glucose produced approximately the same cumulative amount of evolved radioactivity after 140 hours as the ^{14}C L-glucose; however, the kinetics of evolution were obviously different for the two substrates. The ^{14}C D-glucose ($2\text{ }\mu\text{Ci/ml}$, $2 \times 10^{-4}\text{ M}$) produced over 1,000 cpm in the first two hours and evolution had plateaued after 25 hours. The ^{14}C L-glucose produced less than 100 cpm in the first two hours and evolution did not plateau after more than 50 hours. It would appear that two different substrates are being metabolized. The possibility of inhibition of D-glucose by L-glucose exists, however.

Another possible explanation might have been that soil catalyzed the breakdown of L-glucose to some other assimilable substrate and that the slow kinetics of $^{14}\text{CO}_2$ evolution were controlled by this chemical breakdown. To test this possibility, natural Wyaconda soil was added to the preparation of sterile soil plus ^{14}C L-glucose which had previously been used for control in Figure 10.

Kinetics of the response obtained after this inoculation were similar to those obtained when the ^{14}C L-glucose was added to the viable

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soil. If the sterile soil caused a breakdown of L-glucose into biologically active compounds then one would presume an accumulation of these compounds, after 160 hours, that would produce a rapid response upon inoculation. No rapid response occurred; therefore, it is assumed that chemical breakdown of the L-glucose in the soil did not occur.

As shown in Figure 9, B. subtilus inoculated soil appeared to show a slight positive response to the L-glucose.

UL 14 C Sucrose (Figure 12)

An excellent response ratio was obtained with natural soil; however, the B. subtilus inoculated soil showed no response. This was the only carbohydrate tested to which B. subtilus did not show a response.

1 14 C Lactose (Figure 13)

UL 14 C D-xylose (Figure 14)

UL 14 C Cellulose (Figure 15)

All three of these carbohydrates produced a good viable to sterile ratio of response in the natural soil and the B. subtilus inoculated soil. Cellulose is not soluble in water; therefore, a weighted portion was merely added to the soil surface which was then wet with 0.1 ml of sterile H₂O.

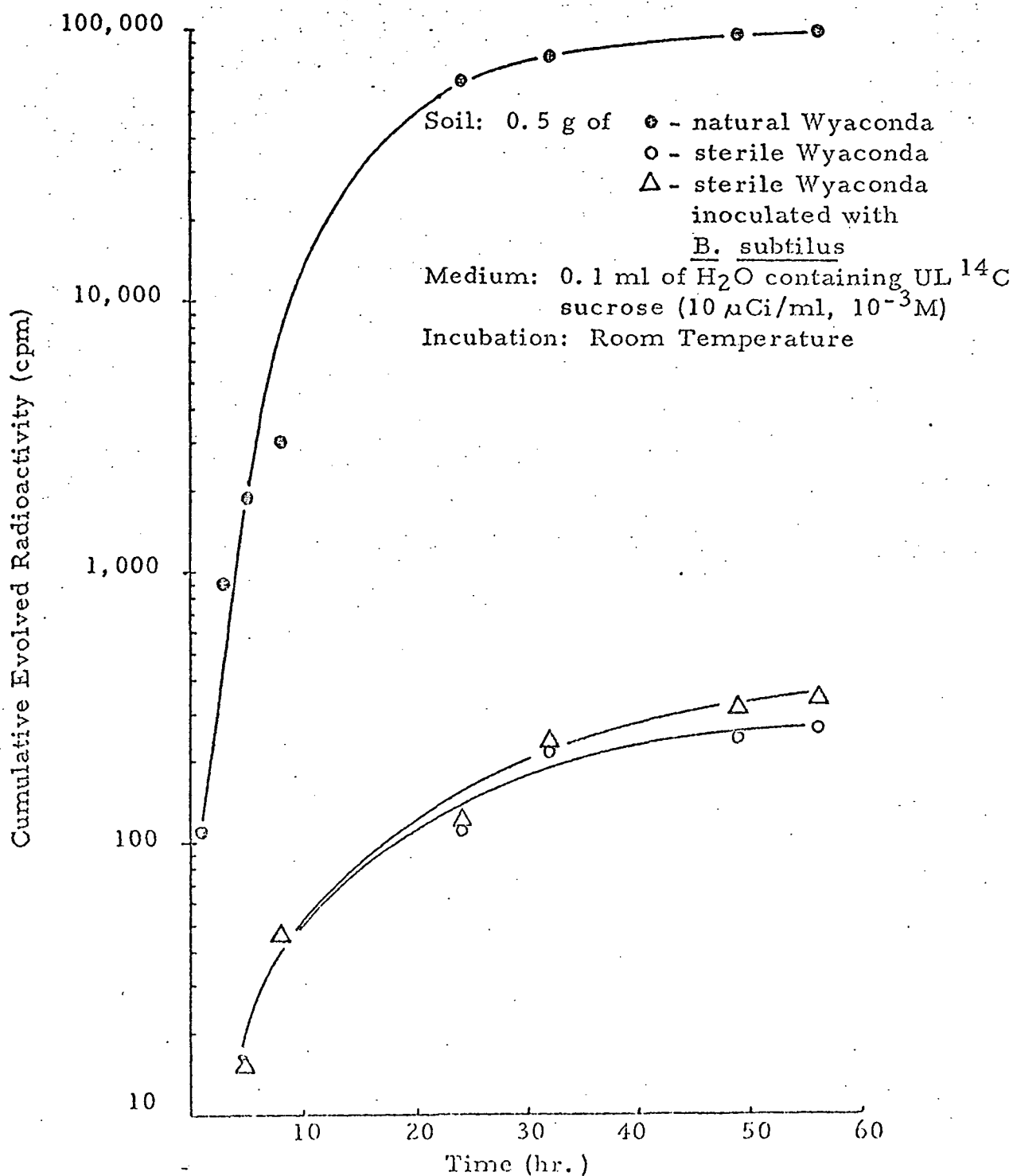
Ring 1 14 C Phenylalanine (Figure 16)

The natural Wyaconda soil showed a response ratio of 100; however, the B. subtilus inoculated soil showed no response.

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FIGURE 12

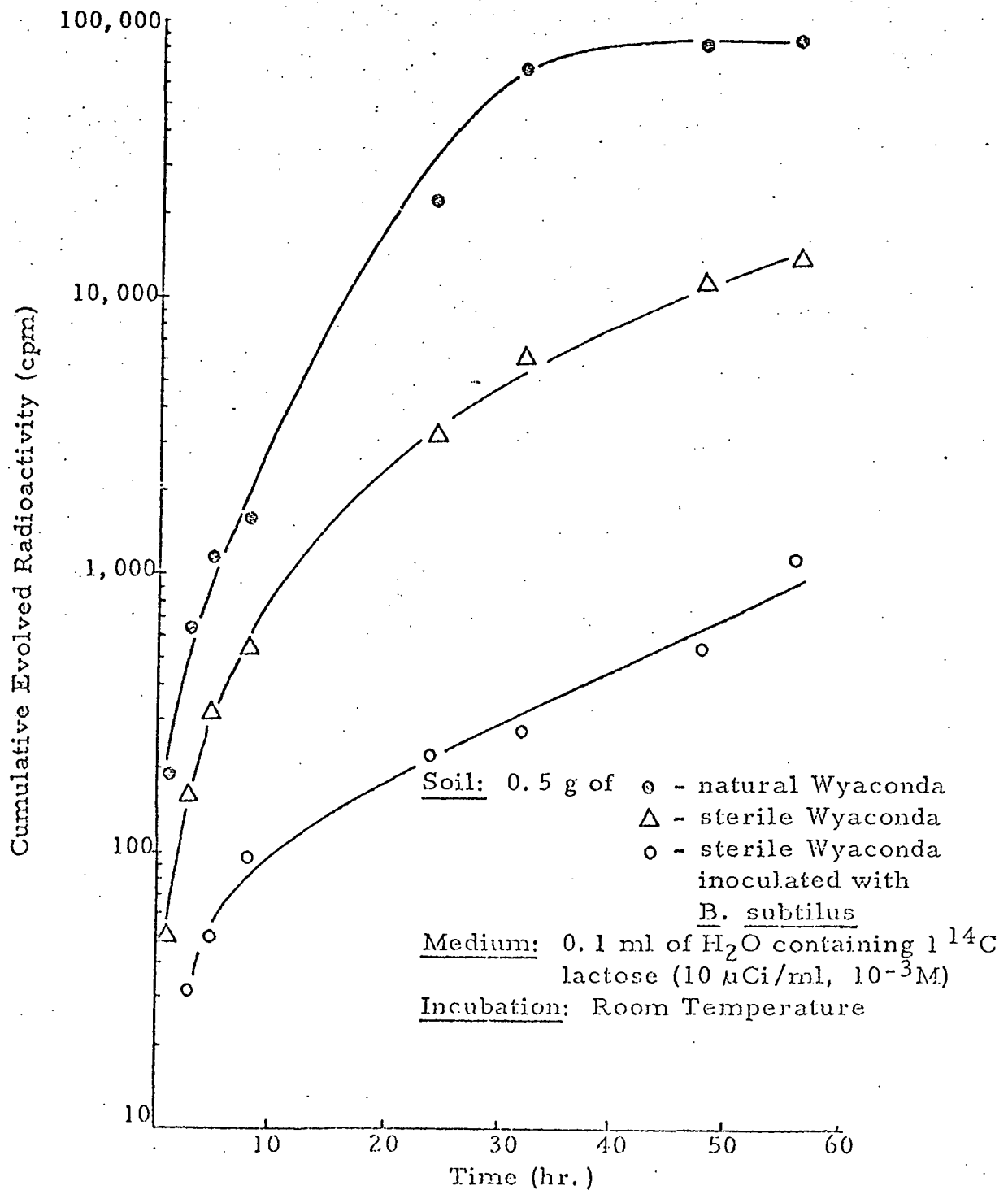
Evolution of $^{14}\text{CO}_2$ from UL ^{14}C Sucrose



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FIGURE 13

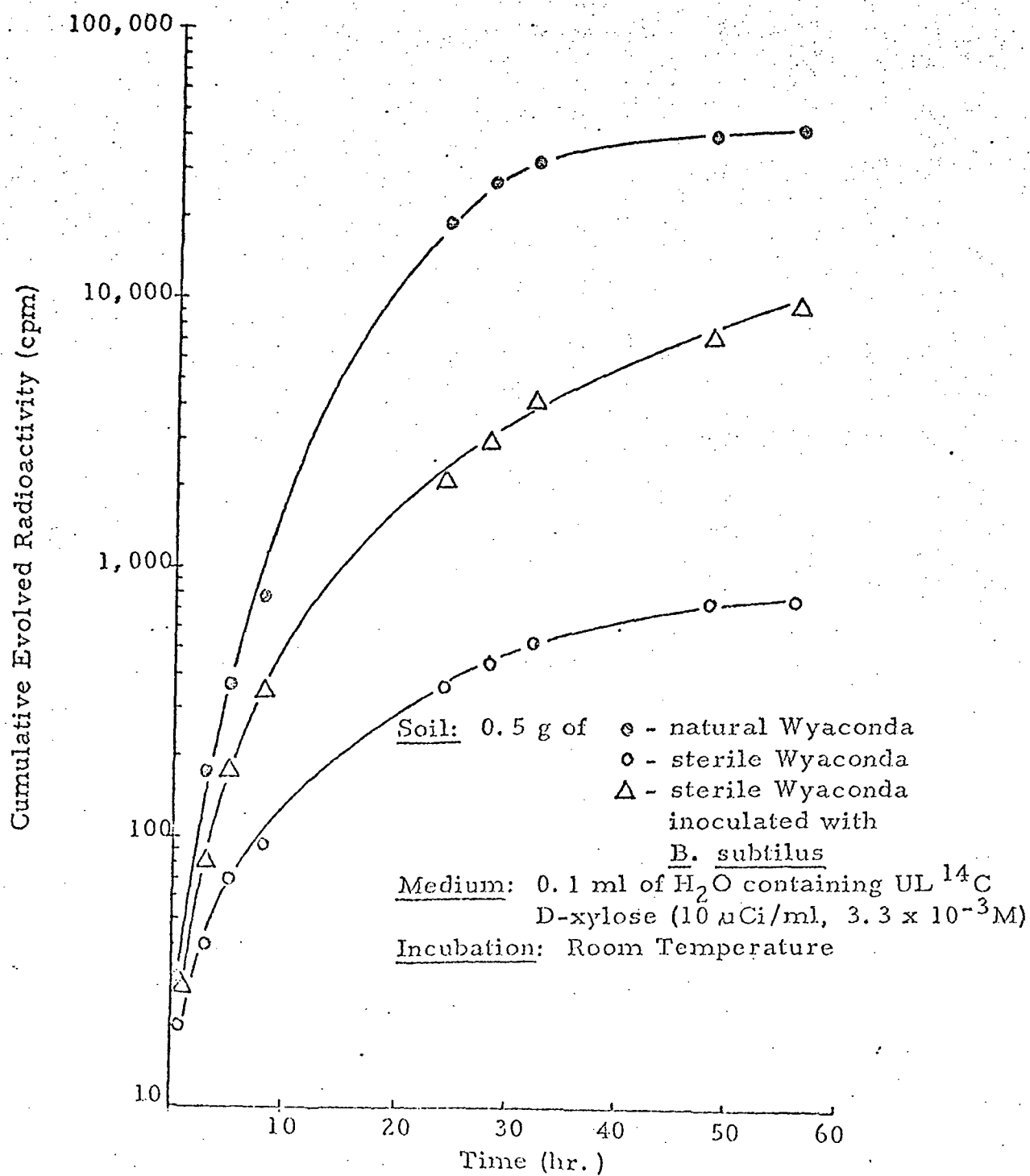
Evolution of $^{14}\text{CO}_2$ from $1\text{ }^{14}\text{C}$ Lactose



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FIGURE 14

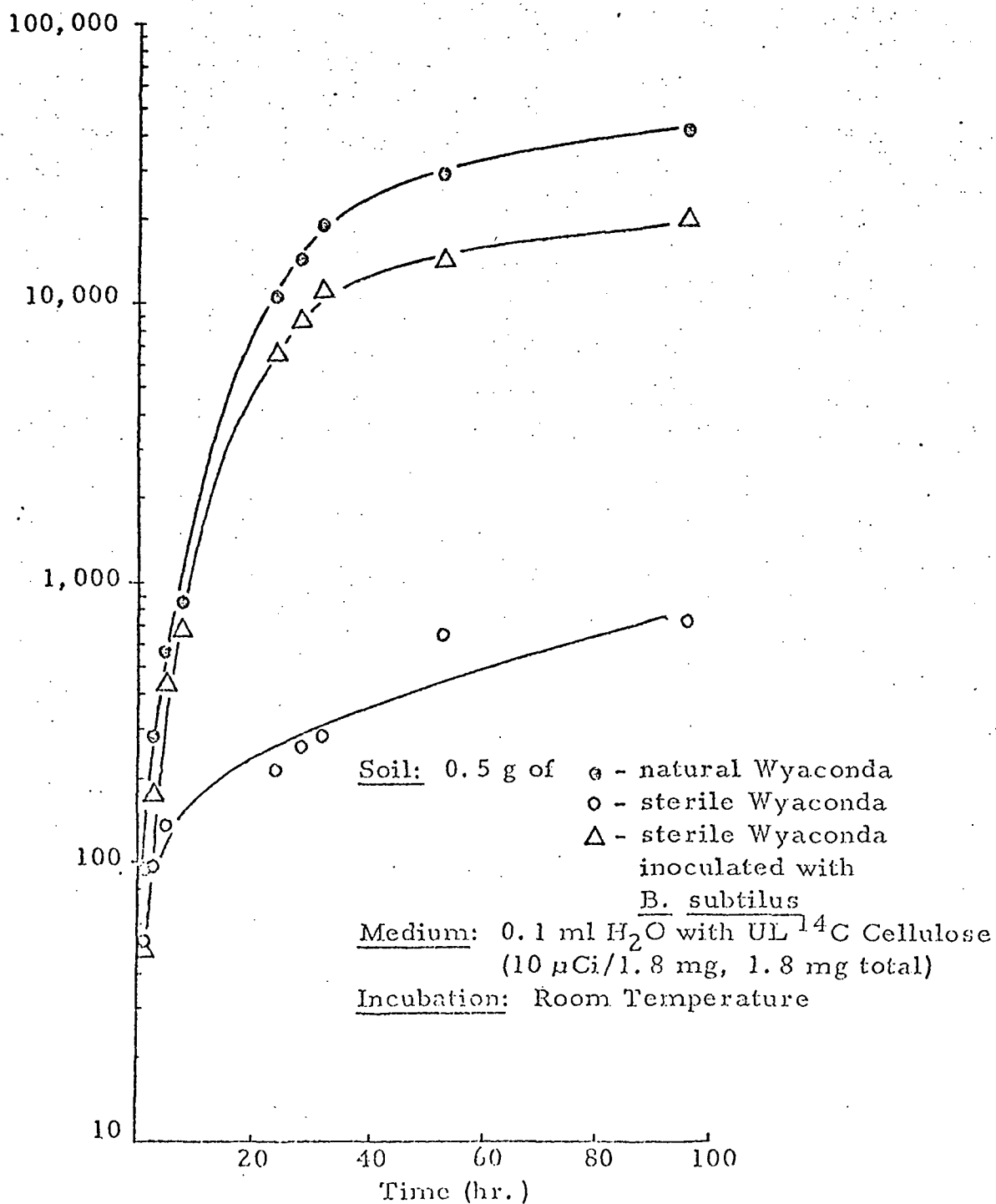
Evolution of $^{14}\text{CO}_2$ from UL ^{14}C D-Xylose



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FIGURE 15

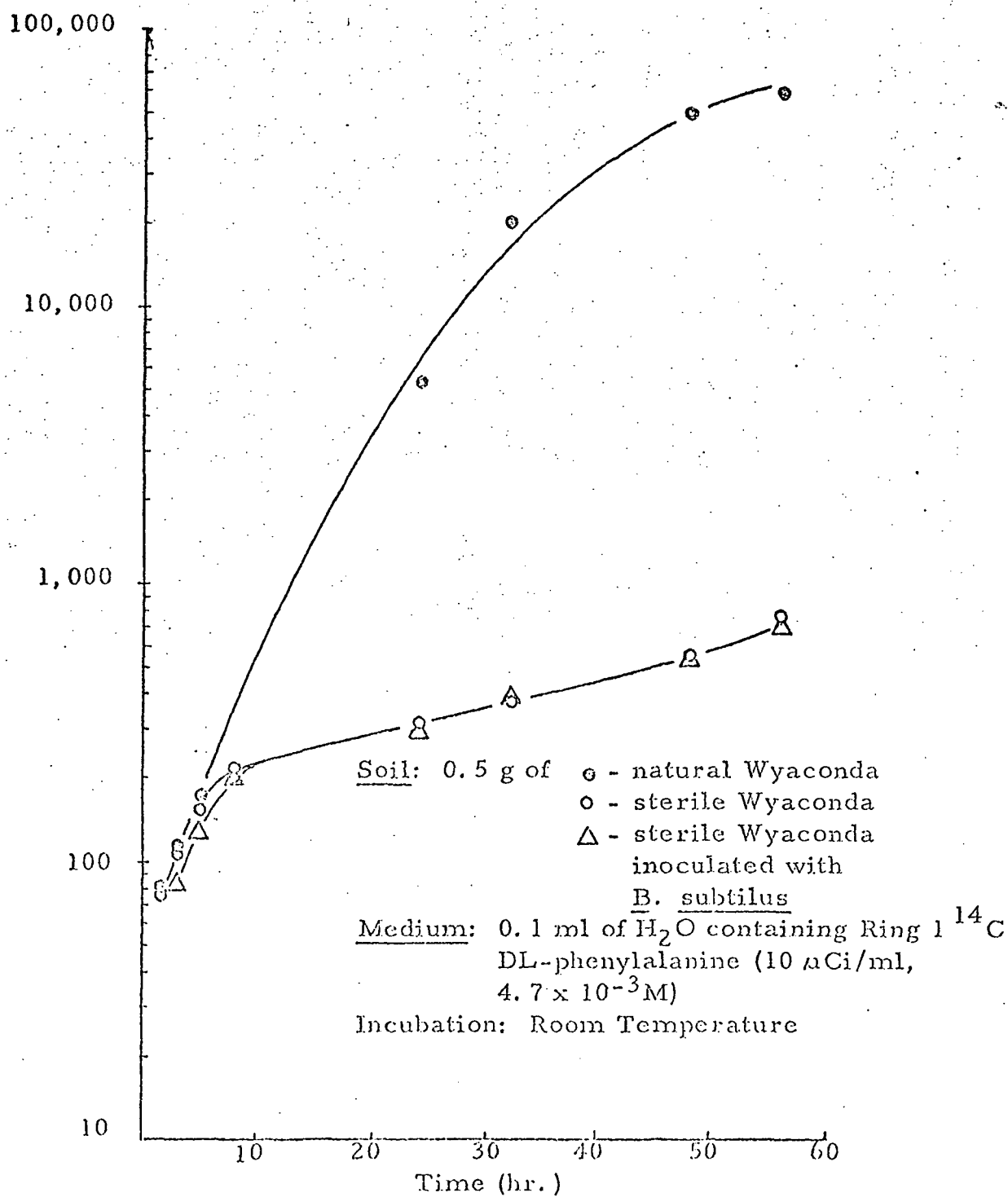
Evolution of $^{14}\text{CO}_2$ from UL ^{14}C Cellulose



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FIGURE 16

Evolution of $^{14}\text{CO}_2$ from Ring 1 ^{14}C DL-Phenylalanine



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$$\frac{1 \text{ } ^{14}\text{C L-valine (Figure 17)}}{1 \text{ } ^{14}\text{C DL-valine}}$$

Both isomers of valine produced a similar response to natural Wyaconda soil. Soil inoculated with B. subtilus showed similar results to both L-valine and DL-valine, but these responses were not nearly as great as those from the natural soil. The DL-valine sterile control was higher than the L-valine control and it appears to be this control level which produced a difference in the response ratios.

$$\frac{1 \text{ } ^{14}\text{C DL-methionine (Figure 18)}}{1 \text{ } ^{14}\text{C DL-methionine}}$$

The relatively high sterile control reduced the response ratio for this compound.

$$\frac{\text{UL } ^{14}\text{C Glycine (Figure 19)}}{\text{UL } ^{14}\text{C Glycine}}$$

The natural Wyaconda soil produced a strong response to glycine; however, the B. subtilus inoculated soil evolved only slightly more radioactivity than the sterile control.

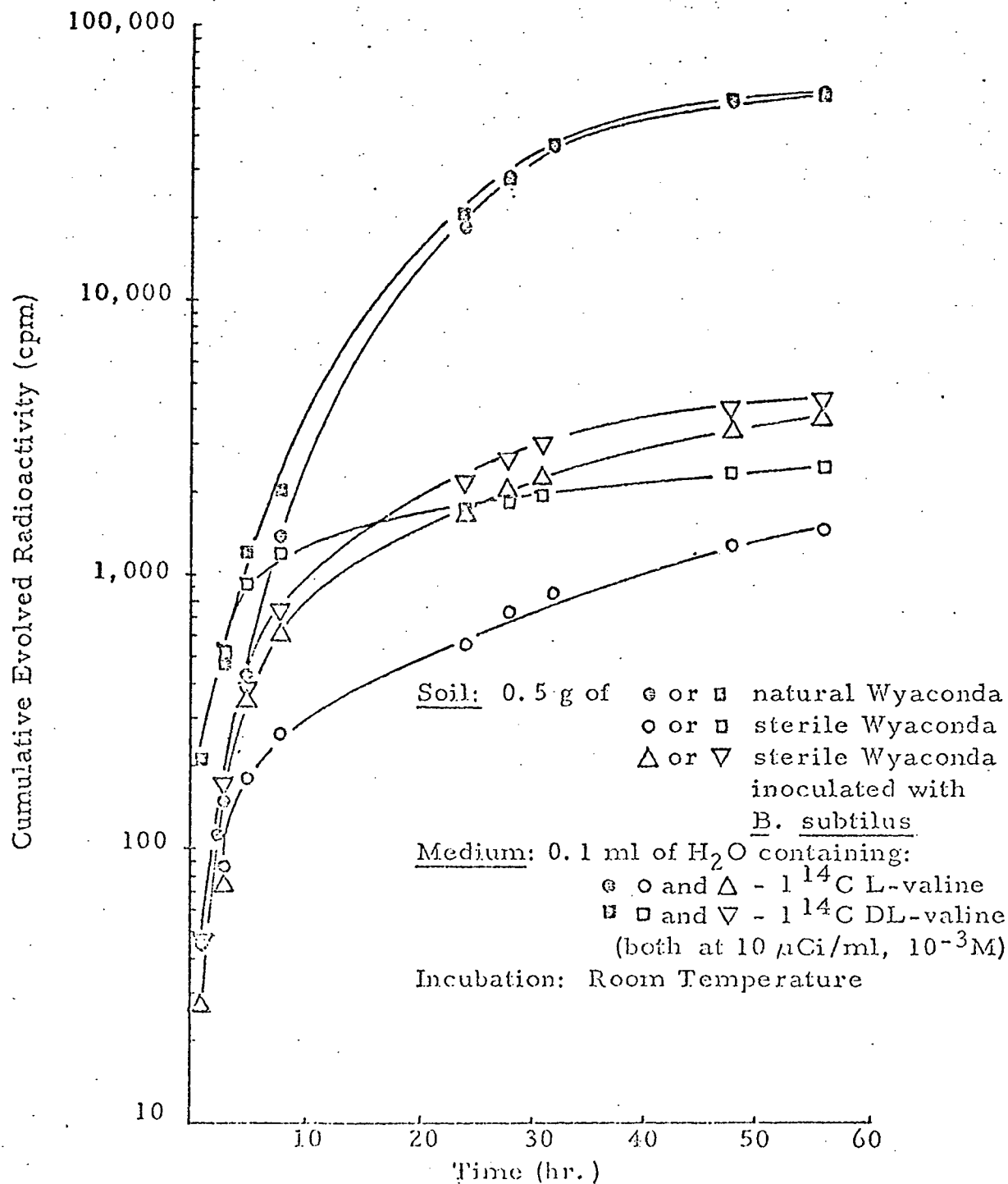
$$\frac{\text{UL } ^{14}\text{C D-alanine (Figure 20)}}{\text{UL } ^{14}\text{C L-alanine}}$$

Natural Wyaconda soil evolved similar total cumulative radioactivities from these isomers although it appeared that L-alanine produced a more rapid response. Sterilized controls for both D- and L-alanine displayed approximately the same level of nonbiological activity. There was considerable difference in the response obtained from the soil inoculated with B. subtilus. The L-alanine appeared to be utilized

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FIGURE 17

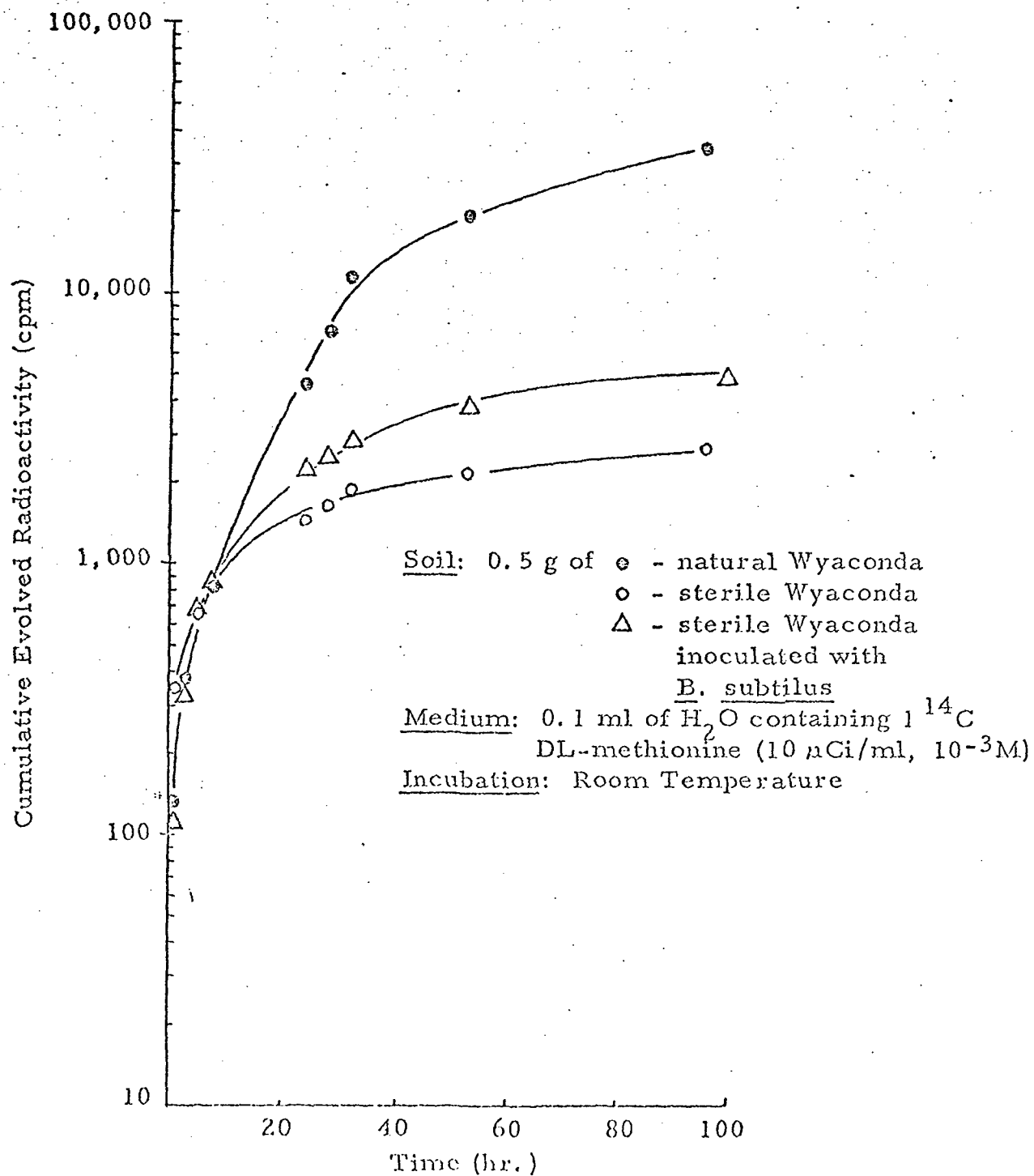
Evolution of $^{14}\text{CO}_2$ from $1\text{ }^{14}\text{C}$ L-valine
and $1\text{ }^{14}\text{C}$ DL-valine



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FIGURE 18

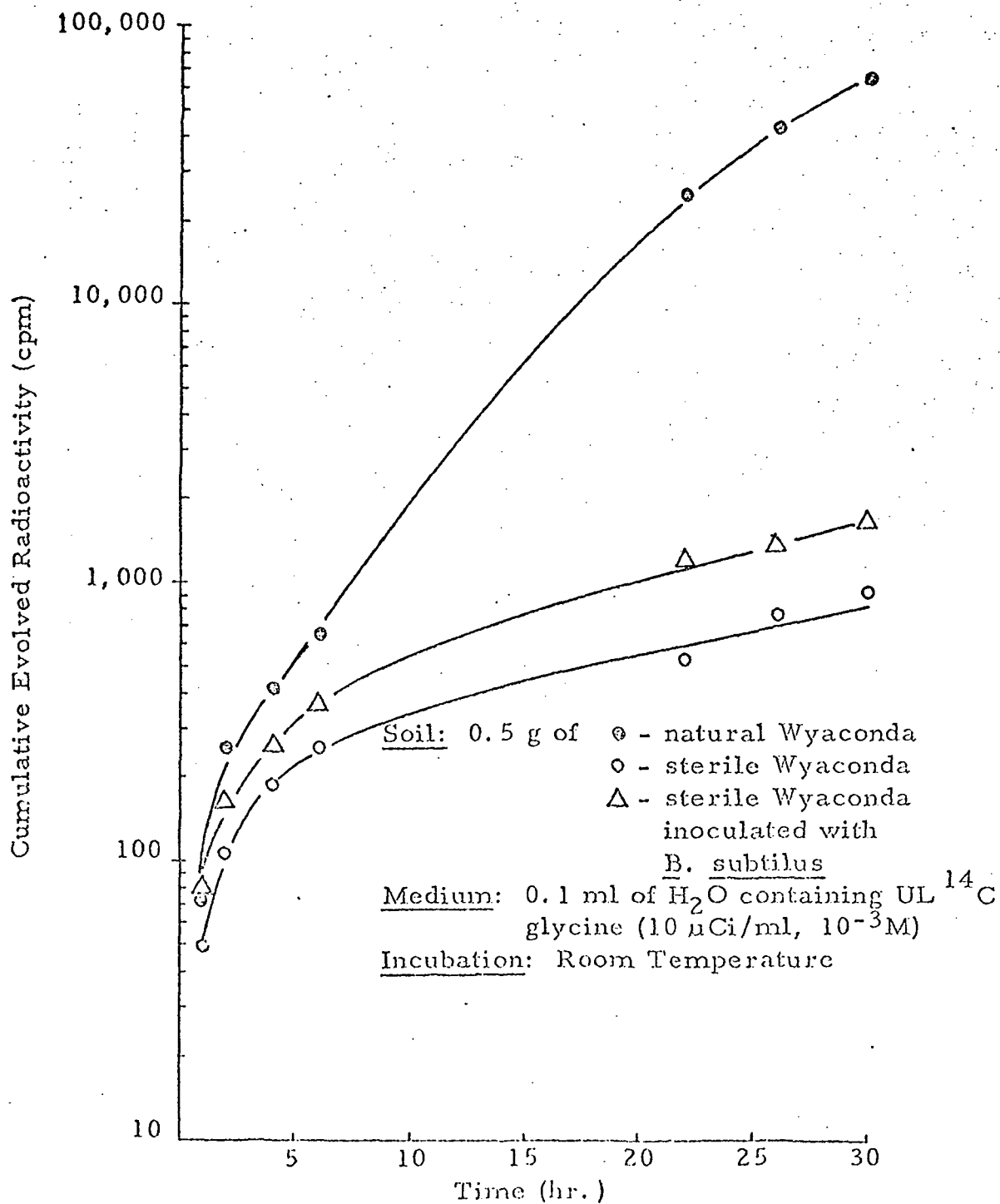
Evolution of $^{14}\text{CO}_2$ from 1^{14}C DL-Methionine



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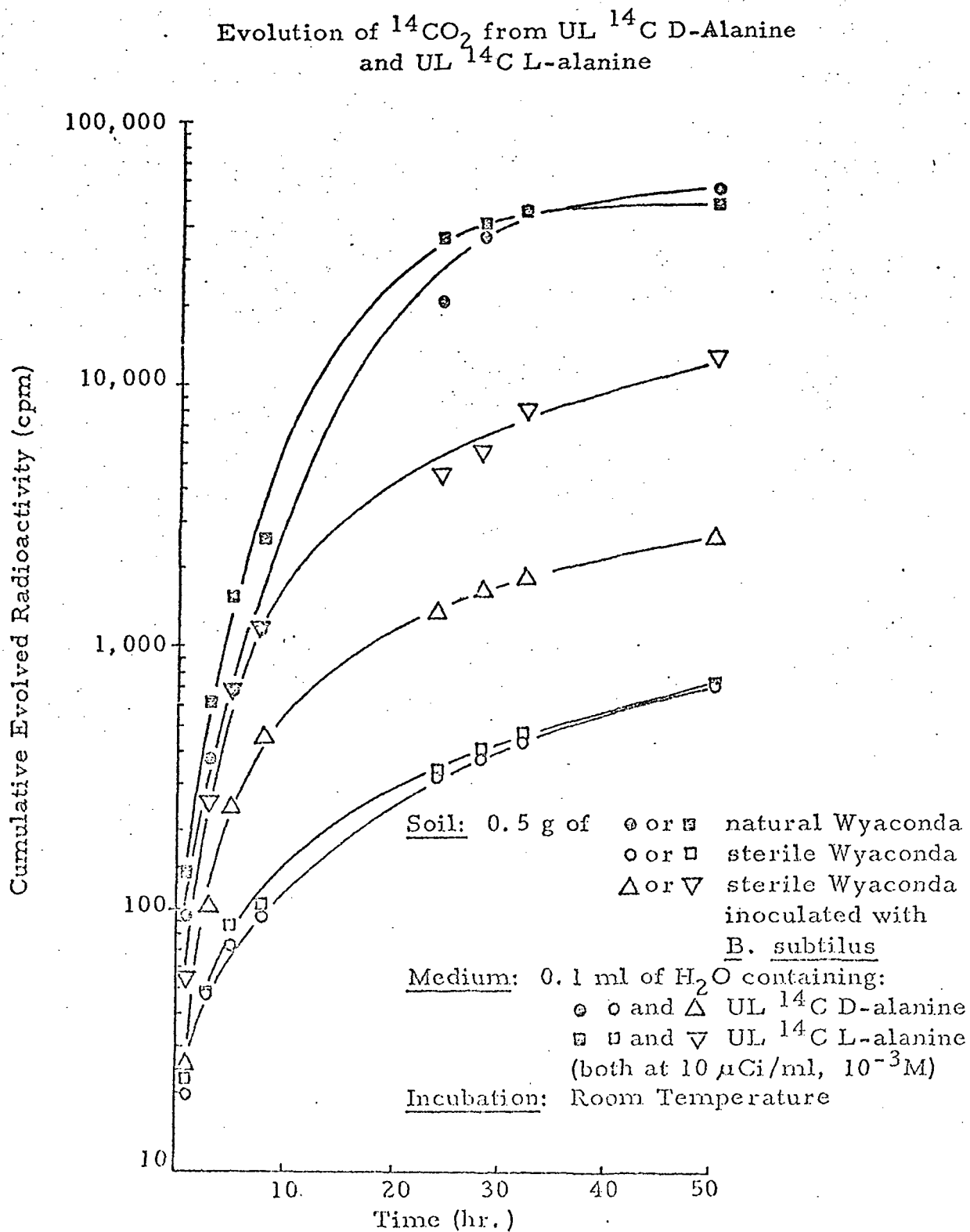
FIGURE 19

Evolution of $^{14}\text{CO}_2$ from UL ^{14}C Glycine



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FIGURE 20



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much more rapidly than the D-alanine with a higher resultant evolution of $^{14}\text{CO}_2$. The response ratios for these two compounds were 18 and 4, respectively.

1 ^{14}C Citric Acid (Figure 21)

A good response ratio for natural Wyaconda soil was obtained; however, the B. subtilus inoculated soil showed no response.

UL ^{14}C α -ketoglutaric Acid (Figure 22)

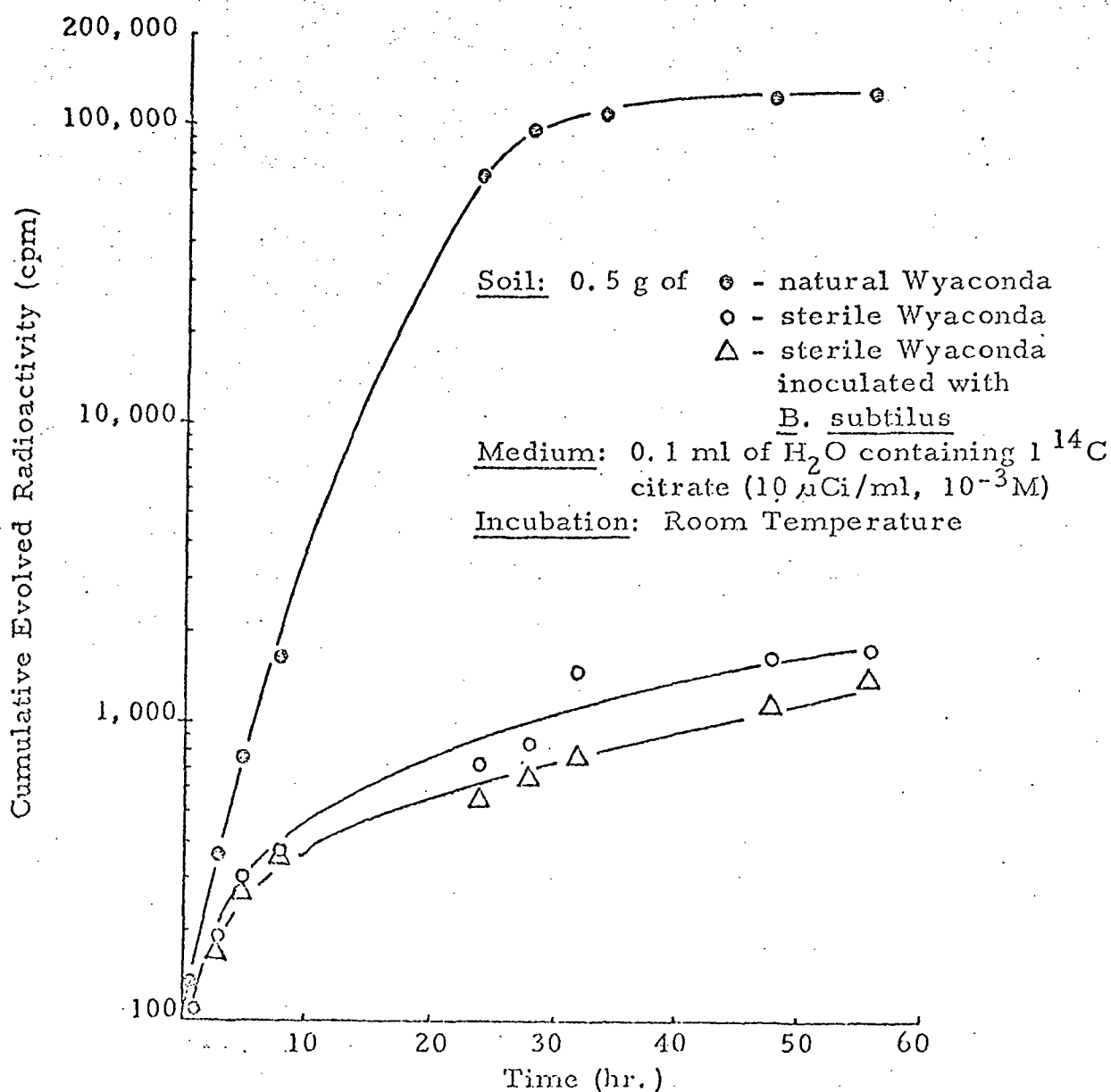
A high sterile control caused this compound to show the lowest response ratio to natural Wyaconda soil, of all the substrates tested. The B. subtilus inoculated soil produced a response considerably less than the sterile soil except after approximately 40 hours of incubation. This phenomenon, where the B. subtilus soil was less than the sterile control during the early stages of incubation was observed for studies on DL-valine, and a possible explanation may lie in the treatment of the soil. Chemical reactions may occur during heat sterilization of the soil which later cause degradation of some substrates. However, the wetting and subsequent air drying of this sterile soil may act to lessen the destructive effect.

The response ratio (viable/sterile) for the B. subtilus soils was calculated for each substrate tested. These ratios, along with an arbitrary designation of activity against that substrate, are presented in Table 3.

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FIGURE 21

Evolution of $^{14}\text{CO}_2$ from 1 ^{14}C Citrate



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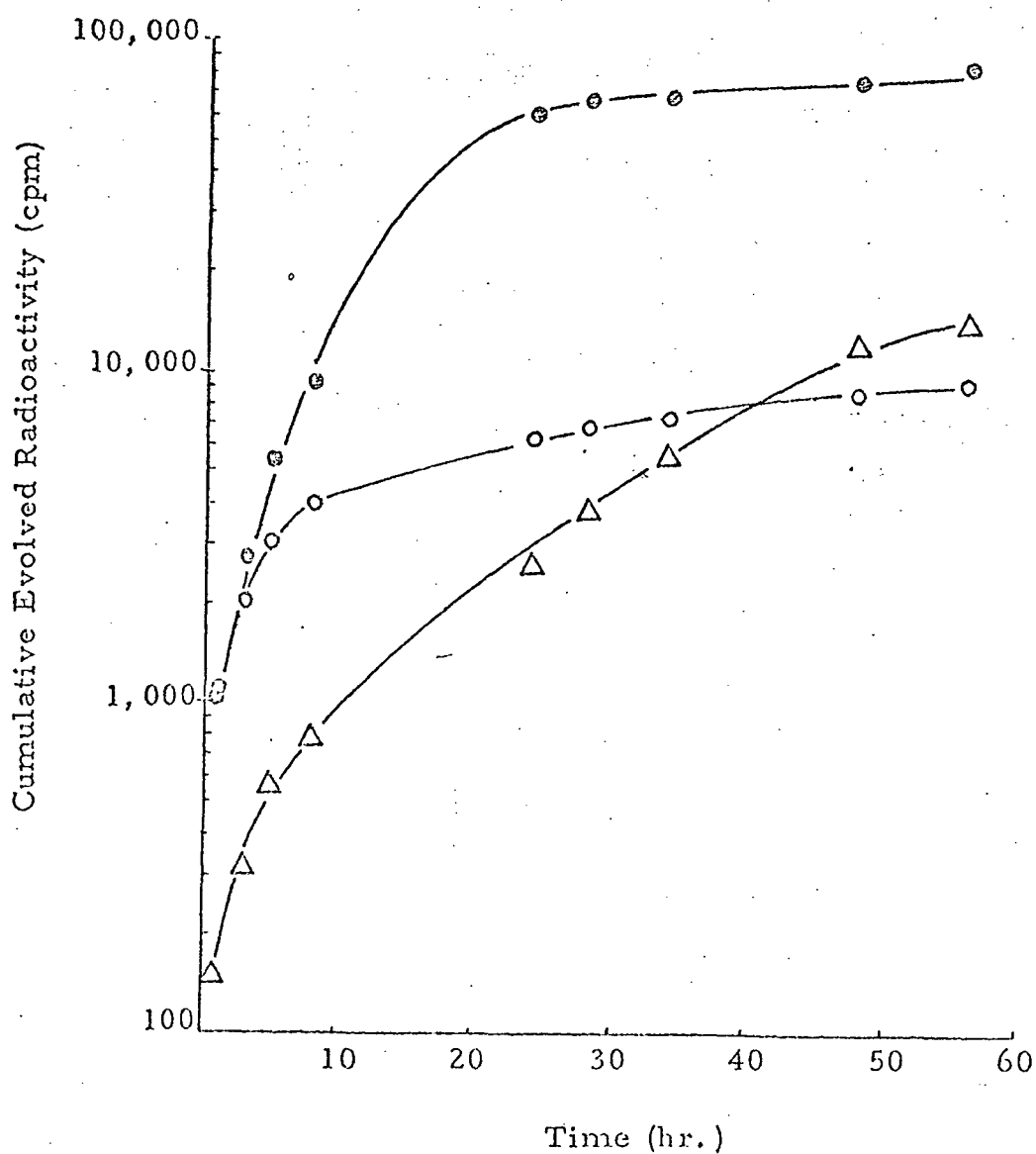
FIGURE 22

Evolution of $^{14}\text{CO}_2$ from UL ^{14}C Alpha-ketoglutarate

Soil: 0.5g of \bullet - natural Wyaconda soil
 \circ - sterile Wyaconda soil
 Δ - sterile Wyaconda soil inoculated
with B. subtilis

Medium: 0.1 ml of H_2O containing UL ^{14}C α ketoglutarate
($10 \mu\text{Ci/ml}$, 10^{-3}M)

Incubation: Room Temperature



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TABLE 3

Response Ratios (Viable/Sterile) Obtained for Various Substrates
with B. subtilus Inoculated Soil

<u>Substrate</u>	<u>Sterile Wyaconda Soil Inoculated with B. subtilus</u>	<u>Metabolic Pattern for B. subtilus Inoculated Soil</u>
<u>Sugars</u>		
1 ¹⁴ C D-glucose	300	+
1 ¹⁴ C L-glucose	5	sl
U ¹⁴ C sucrose	1	-
1 ¹⁴ C lactose	23	+
U ¹⁴ C D-xylose	10	+
U ¹⁴ C cellulose	40	+
<u>Amino Acids</u>		
Ring 1 ¹⁴ C D-phenylalanine	1	-
1 ¹⁴ C L-valine	3	sl
1 ¹⁴ C DL-valine	1	-
1 ¹⁴ C DL-methionine	2	sl
U ¹⁴ C Glycine	2	sl
U ¹⁴ C D-alanine	4	sl
U ¹⁴ C L-alanine	18	+
<u>Krebs Cycle</u>		
1 ¹⁴ C citrate	1	-
U ¹⁴ C alpha-ketoglutarate	1	-

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A response ratio of greater than five was considered positive, a ratio of two - five was considered slight and a ratio of ≤ 2 was considered negative. The result produced a profile for the B. subtilus soil as shown in Table 3. Although the list of test substrates is far from complete, the principal of the approach is illustrated. If sufficient substrates from each group had been tested, the following predictions concerning the physiology of the unknown organism(s) might be made:

- o The carbohydrates tested are readily metabolized except for sucrose.
- o The amino acids tested are not actively metabolized with the exception of L-alanine.
- o The Krebs cycle intermediates tested are not metabolized.

A library of activities by various organisms whose physiology is well known needs to be established. Such data on Martian organisms, along with temperature and inhibitor studies, which could follow the same methodological line as for the substrate study, would provide a considerable description and a basis for comparison with terrestrial biochemistry. A great deal of information may be extracted from the results of each test. For example, the above series shows that the organism is capable of metabolizing 5-carbon and 6-carbon monosaccharides, breaking down lactose but not sucrose (indication of a specific enzyme activity) and degrading cellulose. In addition, the optical isomer preference indicates whether or not an evolutionary choice had been made and what it was. But caution must

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be exercised in attempting to draw conclusions concerning the pathways of metabolism which may exist. Several factors other than the presence or absence of specific pathways may strongly affect results. Cell permeability, active transport and, perhaps, the existence of extracellular enzymes may determine whether a specific substrate may actually be available for disassembly by the cell "machinery". Alternate routes of metabolism may exist which are influenced by environmental conditions and the physiological state of the organisms. Some of these alternate routes may produce CO_2 while others do not. Some amino acids may be fermented with the production of CO_2 or they may be incorporated into stable cellular protein. Oxidation as opposed to fermentation may also occur. The mere fact that $^{14}\text{CO}_2$ is evolved from ^{14}C labeled citrate does not constitute evidence for the citric acid cycle. The production of $^{14}\text{CO}_2$ from labeled substrates in conjunction with inhibitor blocked pathways may contribute more specific information. In addition, the ^{14}C -organic uptake experiment may clarify interpretation.

2. Combined Substrate Addition

Three media were prepared which contained all the substrates in a chemical category, which had been tested individually. The media contained the following:

Carbohydrate Medium

1 ^{14}C D-glucose, 1 ^{14}C L-glucose
UL ^{14}C sucrose, 1 ^{14}C lactose all $2 \times 10^{-4}\text{M}$, 2 $\mu\text{Ci/ml}$
UL ^{14}C D-xylose, $6.6 \times 10^{-4}\text{M}$, 2 $\mu\text{Ci/ml}$

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Amino Acid Medium

1 ^{14}C L-valine, 1 ^{14}C D-valine, 1 ^{14}C DL-methionine,
UL ^{14}C glycine, UL ^{14}C D-alanine, UL ^{14}C L-alanine
all 1.43×10^{-4} M, $1.43 \mu\text{Ci/ml}$.
Ring 1 ^{14}C DL-phenylalanine 6.72×10^{-4} M, $1.43 \mu\text{Ci/ml}$.

Krebs Cycle Medium

1 ^{14}C citrate, UL ^{14}C alpha-ketoglutarate both 5×10^{-4} M,
 $5 \mu\text{Ci/ml}$.

The experiments were conducted in aluminum planchets using 0.5 g of natural Wyaconda, sterile Wyaconda and B. subtilus inoculated Wyaconda soil and 0.1 ml of medium.

Results are shown in Figures 23, 24 and 25. It appears that the combination of citrate and alpha-ketoglutarate produced a stronger response from natural Wyaconda soil than the other two groups--carbohydrates and amino acids. On the other hand, only carbohydrates produced a significant response from the B. subtilus inoculated soil.

3. Multiple Addition

The same series of substrates which were tested individually and combined were also tested in the multiple addition mode. The procedure was to add single labeled substrate to natural Wyaconda soil and monitor the evolution of radioactivity. When this evolution reached an apparent plateau, a second addition of soil and medium was made. The concentration and specific activity of the substrates was as given in Table 2. The soil

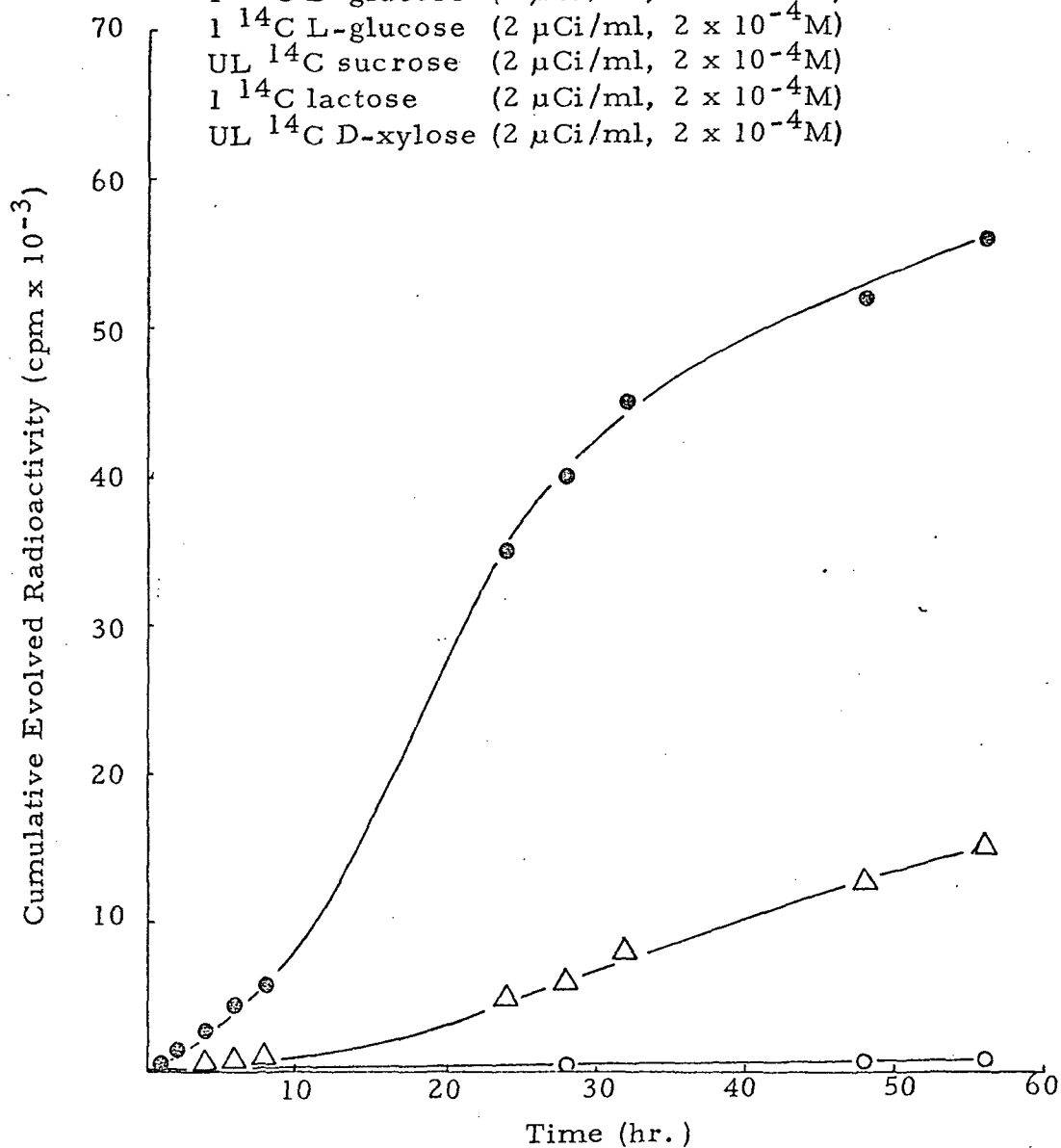
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FIGURE 23

Evolution of $^{14}\text{C}\text{O}_2$ from Medium
Containing ^{14}C Labeled Carbohydrates

Soil: 0.5 g of ● - natural Wyaconda
○ - sterile Wyaconda
△ - sterile Wyaconda
inoculated with B. subtilis

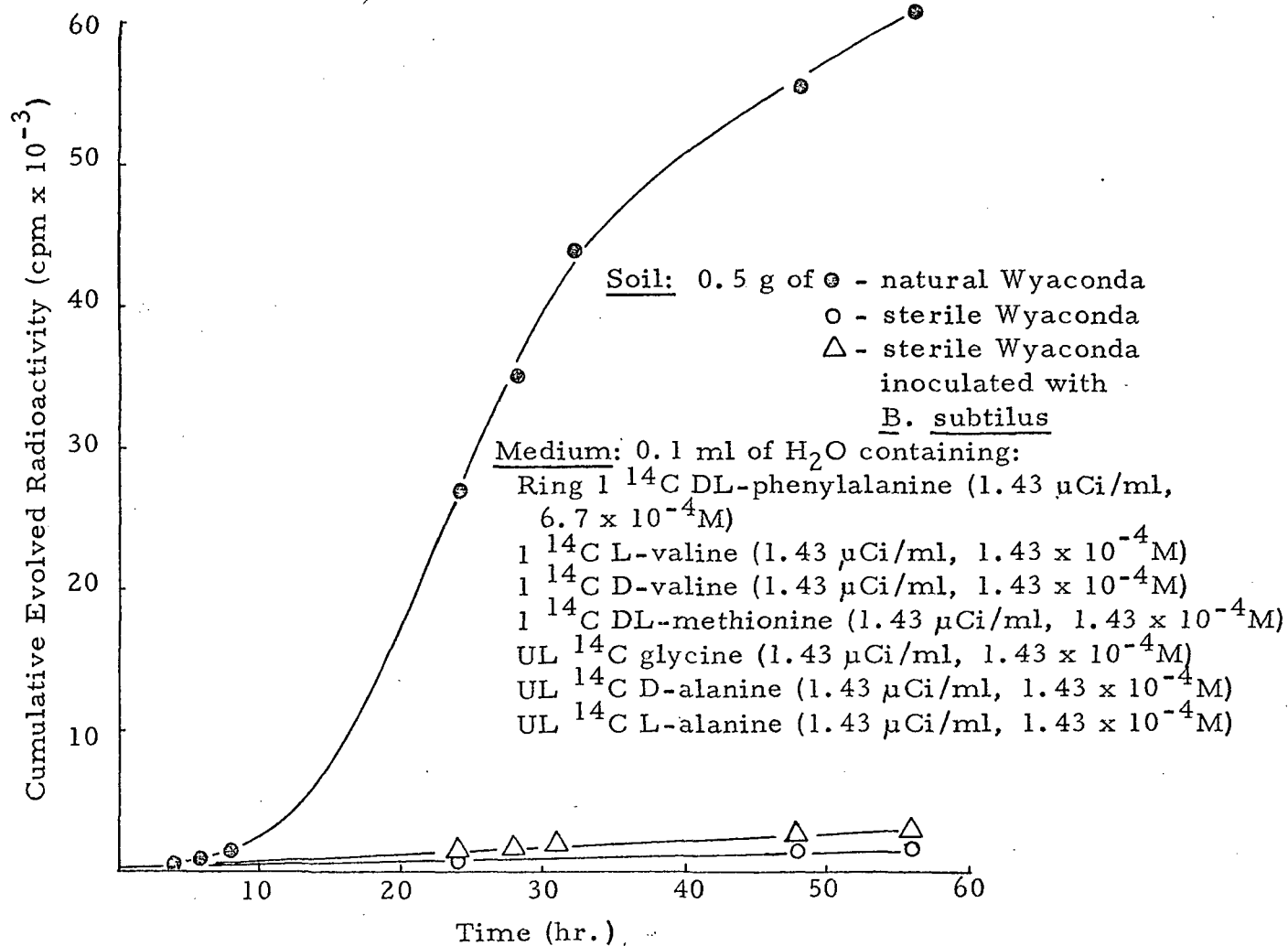
Medium: 0.1 ml of H_2O containing:
1 ^{14}C D-glucose ($2\ \mu\text{Ci/ml}$, $2 \times 10^{-4}\text{M}$)
1 ^{14}C L-glucose ($2\ \mu\text{Ci/ml}$, $2 \times 10^{-4}\text{M}$)
UL ^{14}C sucrose ($2\ \mu\text{Ci/ml}$, $2 \times 10^{-4}\text{M}$)
1 ^{14}C lactose ($2\ \mu\text{Ci/ml}$, $2 \times 10^{-4}\text{M}$)
UL ^{14}C D-xylose ($2\ \mu\text{Ci/ml}$, $2 \times 10^{-4}\text{M}$)



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FIGURE 24

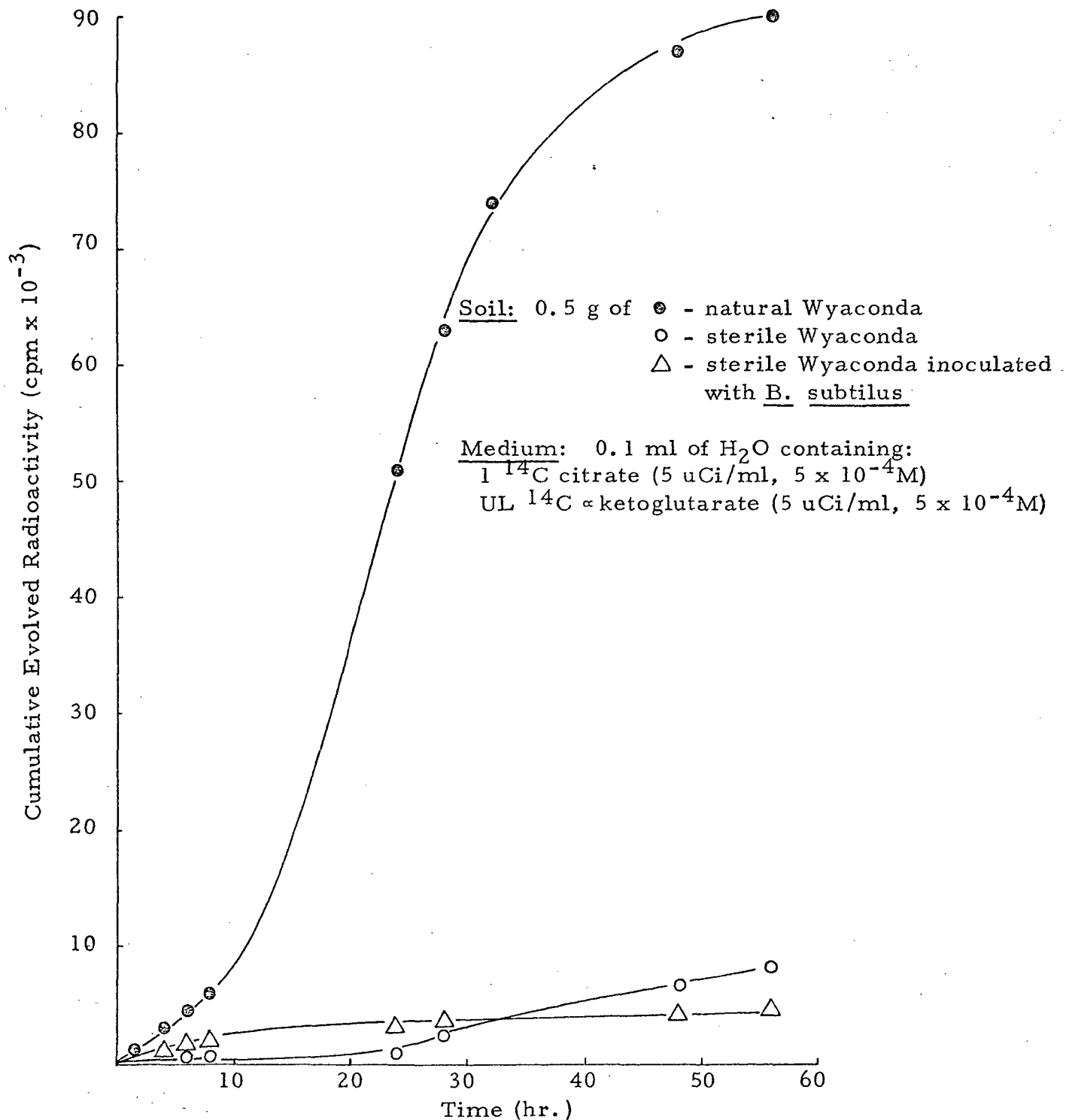
Evolution of $^{14}\text{CO}_2$ from Medium
Containing ^{14}C Labeled Amino Acids



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FIGURE 25

Evolution of $^{14}\text{CO}_2$ from Medium
Containing ^{14}C Labeled Citrate and Alpha-ketoglutarate



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to liquid ratio was 0.5 g/0.1 ml in all cases. The course of $^{14}\text{CO}_2$ evolution and substrate and soil addition was as given in Figures 26 to 37. A separate culture tube was used for each category of substrates.

Figures 26-29 show successive additions of ^{14}C carbohydrates to a single vial. Incubation time and cumulative radioactive evolution are progressive and continuous in these figures. The plateau in $^{14}\text{CO}_2$ evolution from preceeding soil medium additions is shown for each subsequent addition. Figures 30-36 show successive additions of ^{14}C amino acids made to a single vial.

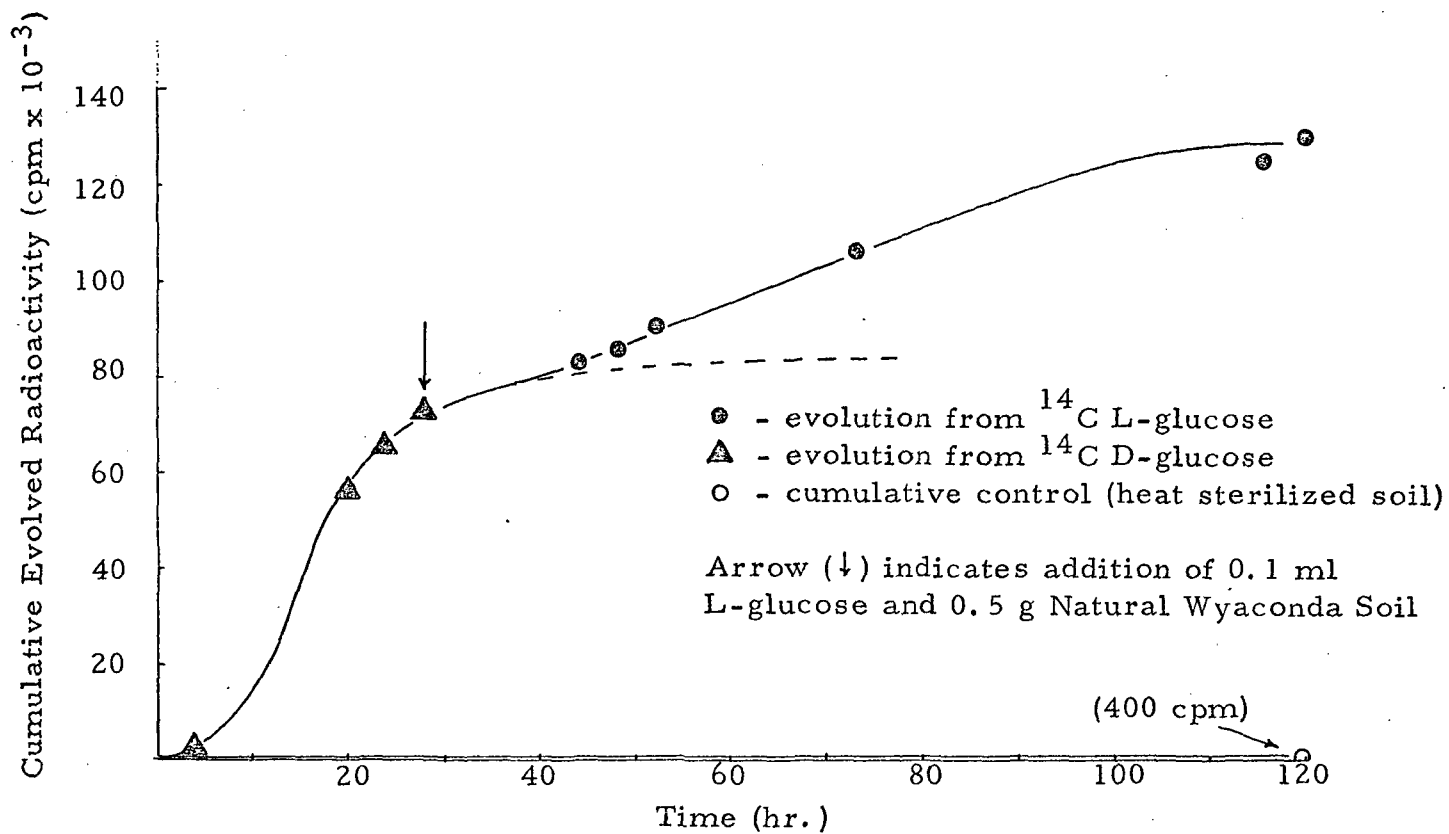
The cumulative evolved radioactivity and maximum viable/sterile response ratio was determined for each substrate addition to natural Wyaconda soil. These calculated values are given in Table 4. Correlation of results obtained with the single and multiple addition modes is good. Both techniques showed a strong response to all substrates tested. There were also indications of a correlation in the magnitude of the response ratio which was obtained. The average response ratio from carbohydrates: Amino Acids: Krebs Cycle was:

Single Addition	150:47:10
Multiple Addition	145:25:25
Combination Single Addition	65:20:20

In both the single addition mode and the multiple addition mode, D-glucose produced the highest response ratio and alpha-ketoglutarate produced the lowest response ratio. The cumulative evolved radioactivity was similar

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FIGURE 26
Evolution of $^{14}\text{CO}_2$ from ^{14}C D-Glucose
and ^{14}C L-Glucose
Multiple Addition Mode



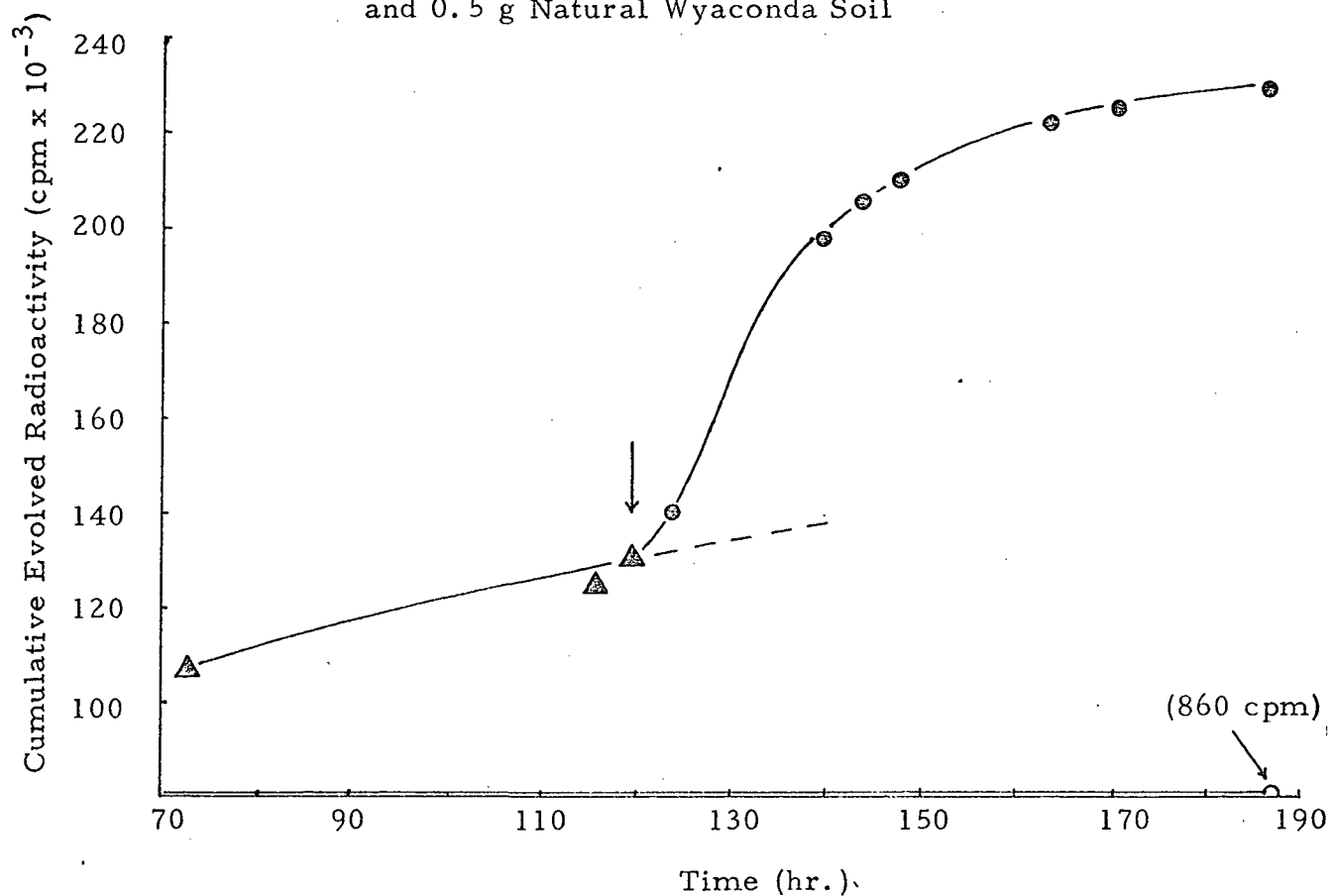
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FIGURE 27

Evolution of $^{14}\text{CO}_2$ from ^{14}C D-Xylose
Multiple Addition Mode

- ⊙ - Evolution from ^{14}C D-xylose
- △ - Evolution from ^{14}C L-glucose
- - Cumulative control (heat sterilized soil)

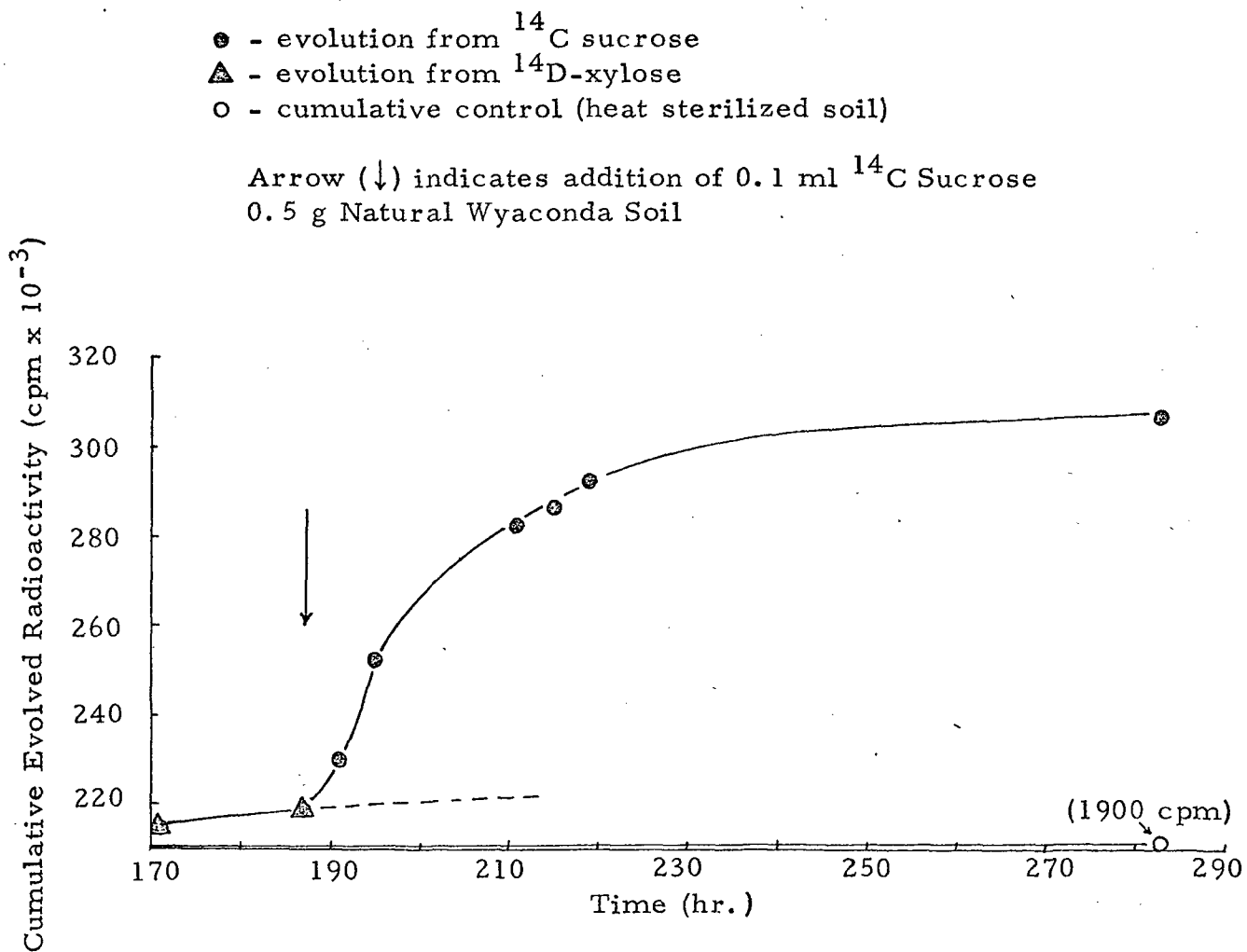
Arrow (↓) indicates addition of 0.1 ml ^{14}C D-xylose
and 0.5 g Natural Wyaconda Soil



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FIGURE 28

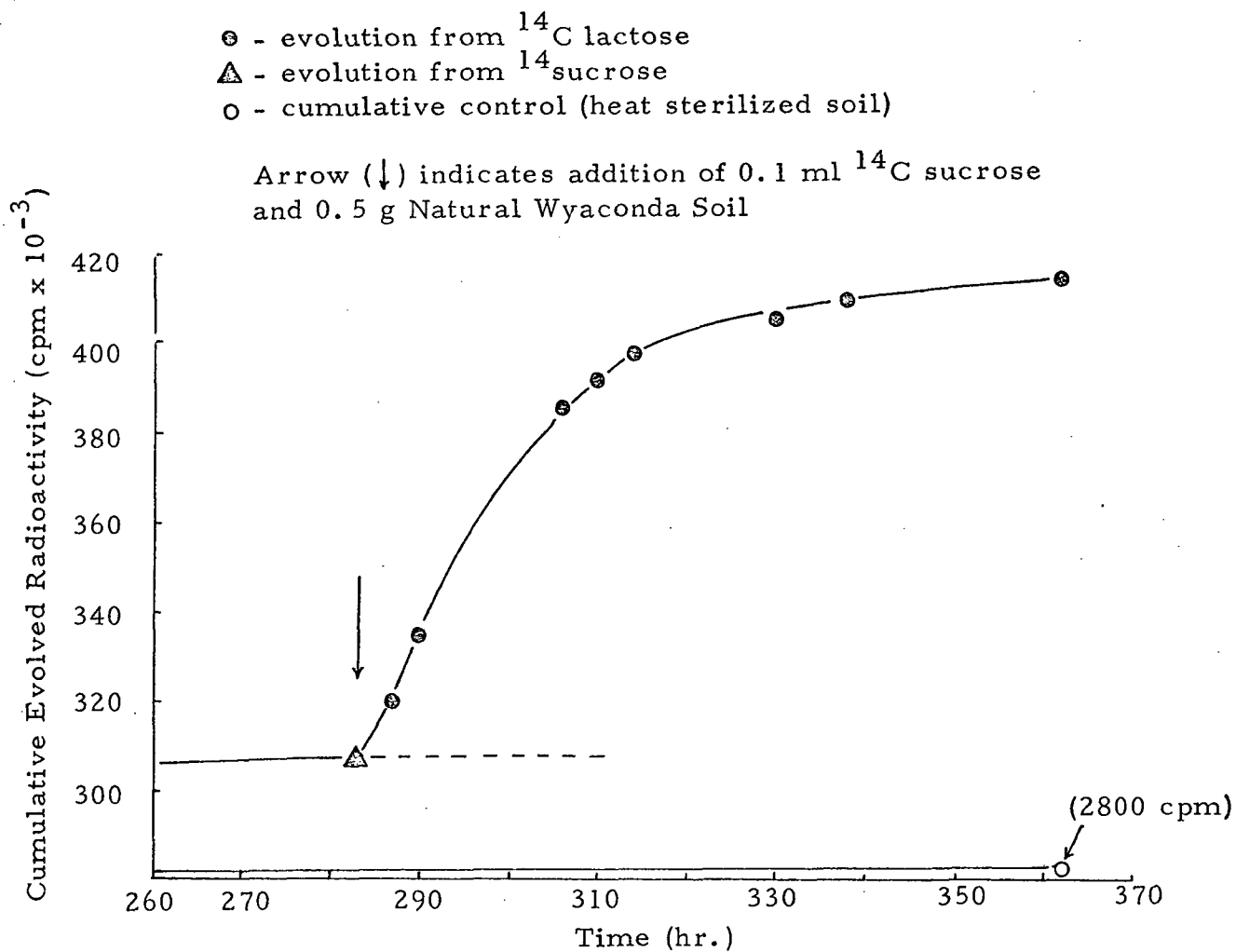
Evolution of $^{14}\text{CO}_2$ from ^{14}C Sucrose
Multiple Addition Mode



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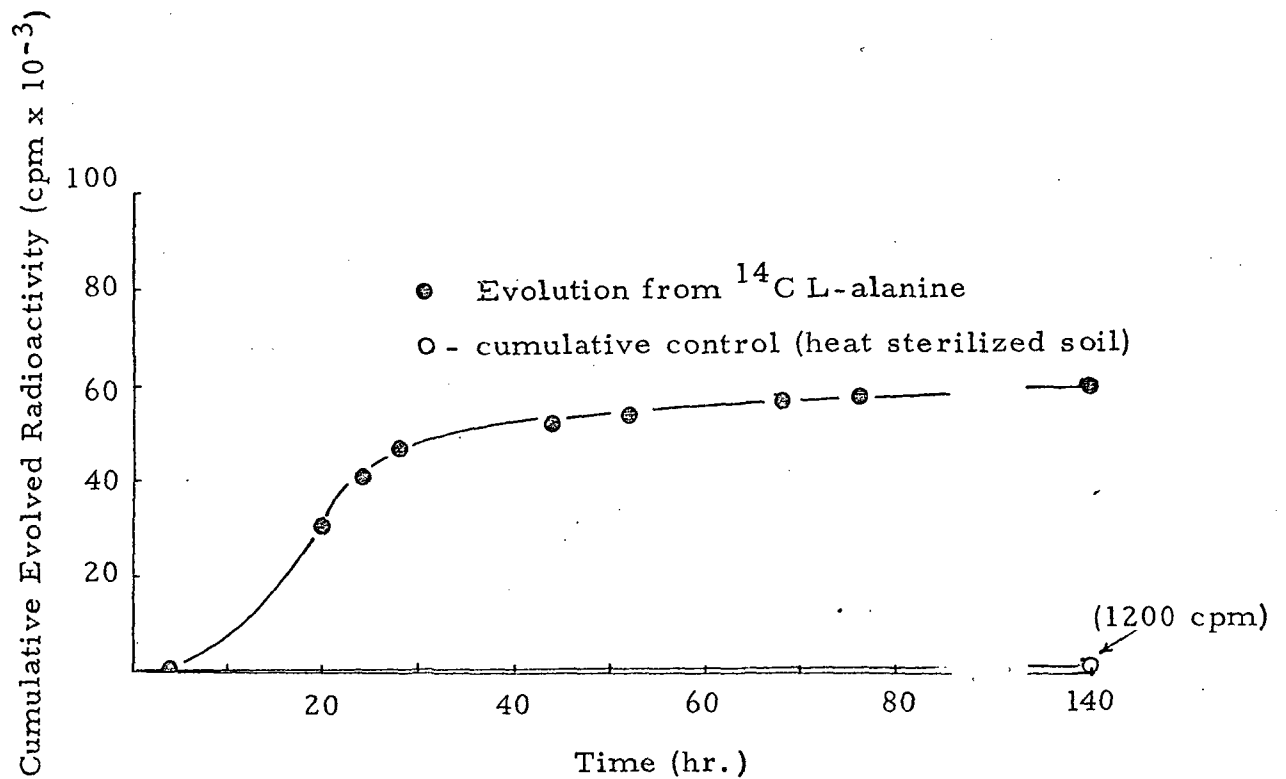
FIGURE 29

Evolution of $^{14}\text{CO}_2$ from ^{14}C Lactose
Multiple Addition Mode



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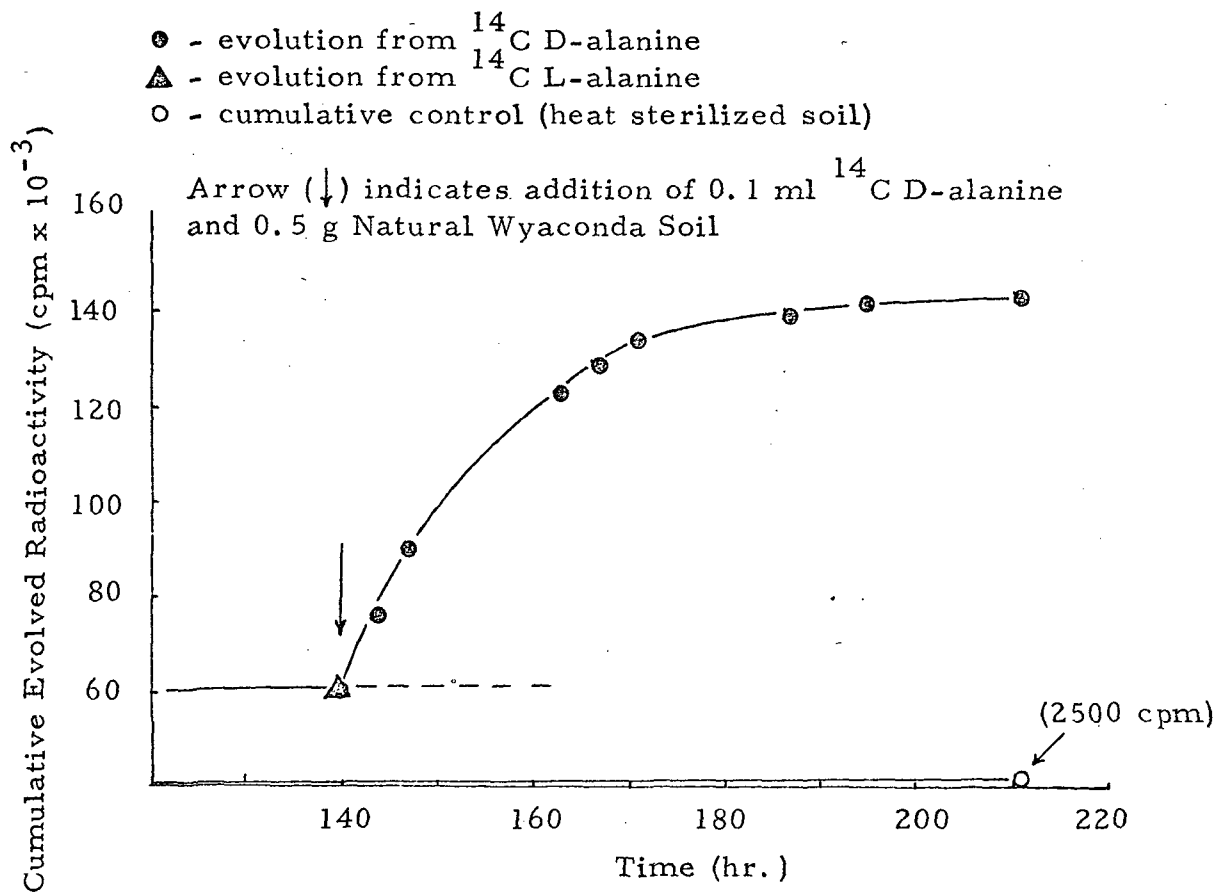
FIGURE 30
Evolution of $^{14}\text{CO}_2$ from ^{14}C L-Alanine
Multiple Addition Mode



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FIGURE 31

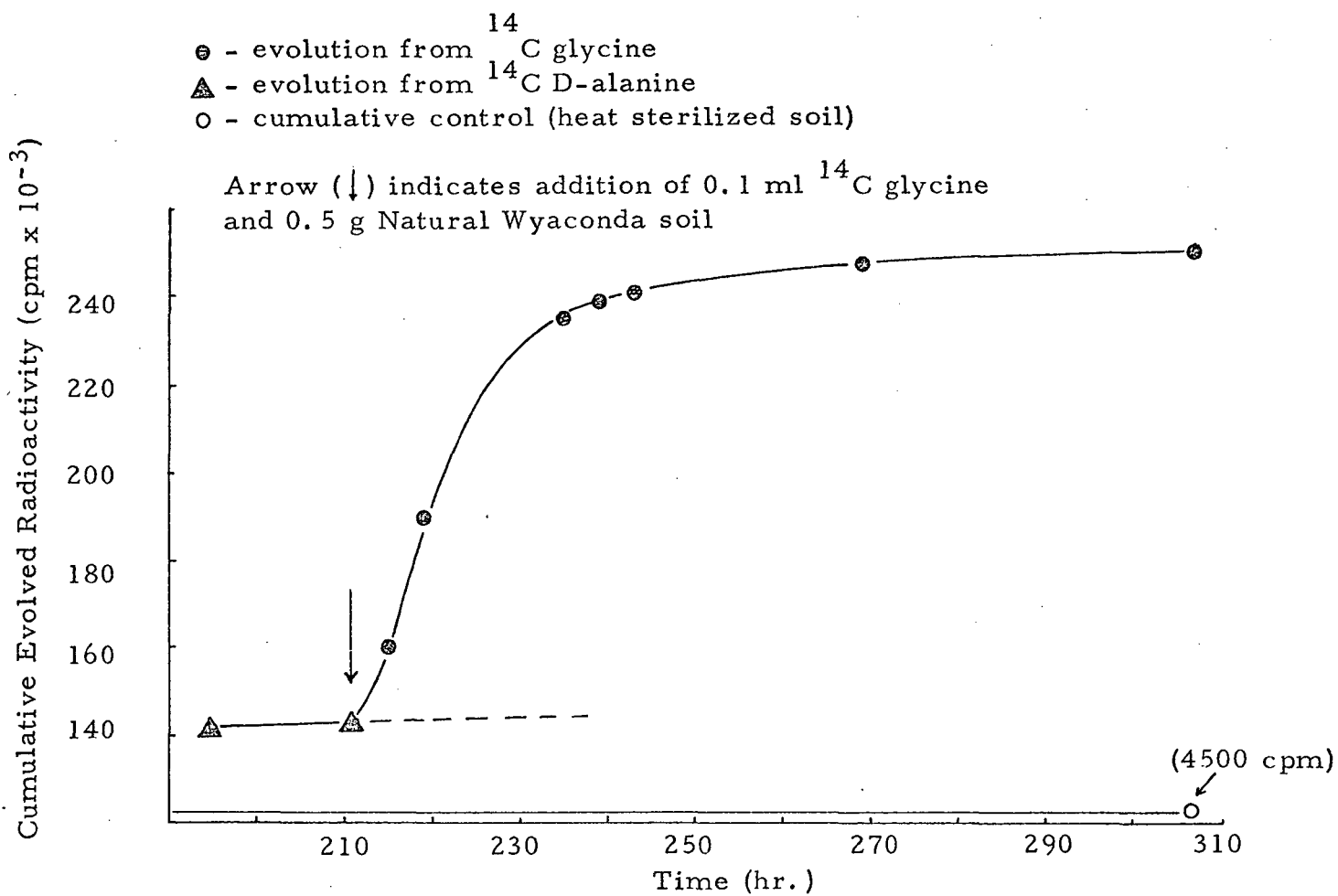
Evolution of $^{14}\text{CO}_2$ from ^{14}C D-Alanine
Multiple Addition Mode



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FIGURE 32

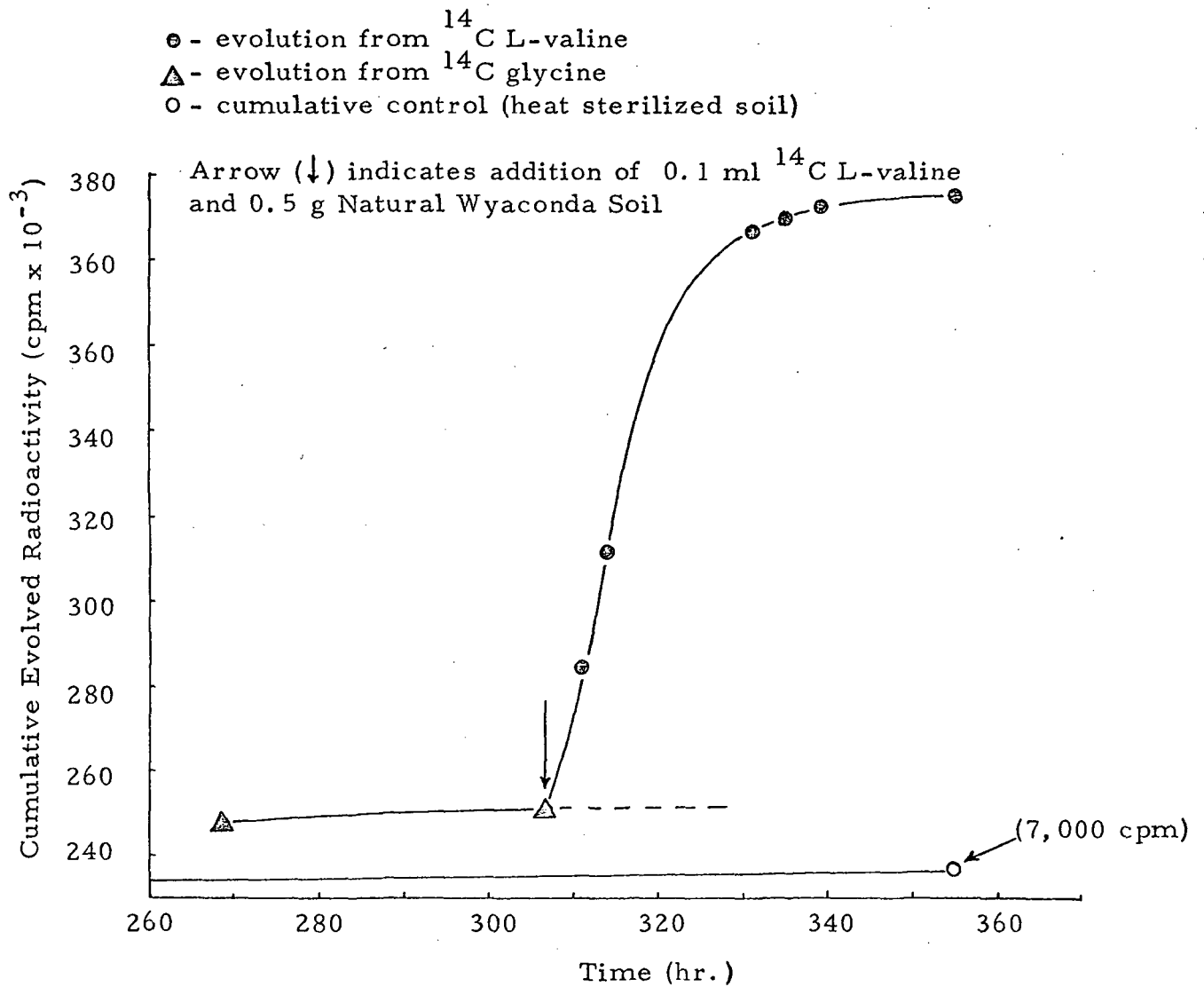
Evolution of $^{14}\text{CO}_2$ from ^{14}C Glycine
Multiple Addition Mode



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FIGURE 33

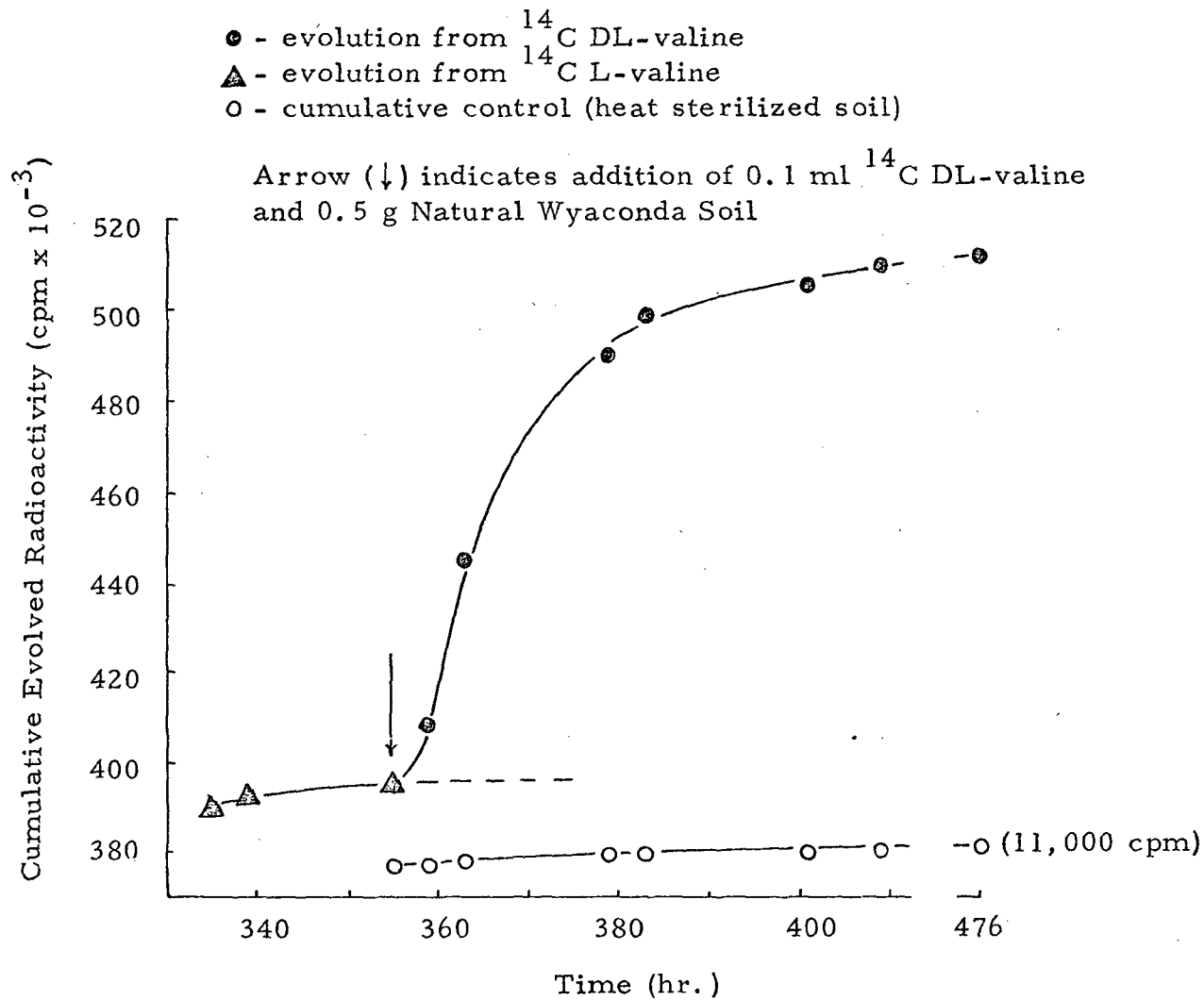
Evolution of $^{14}\text{CO}_2$ from ^{14}C L-Valine
Multiple Addition Mode



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FIGURE 34

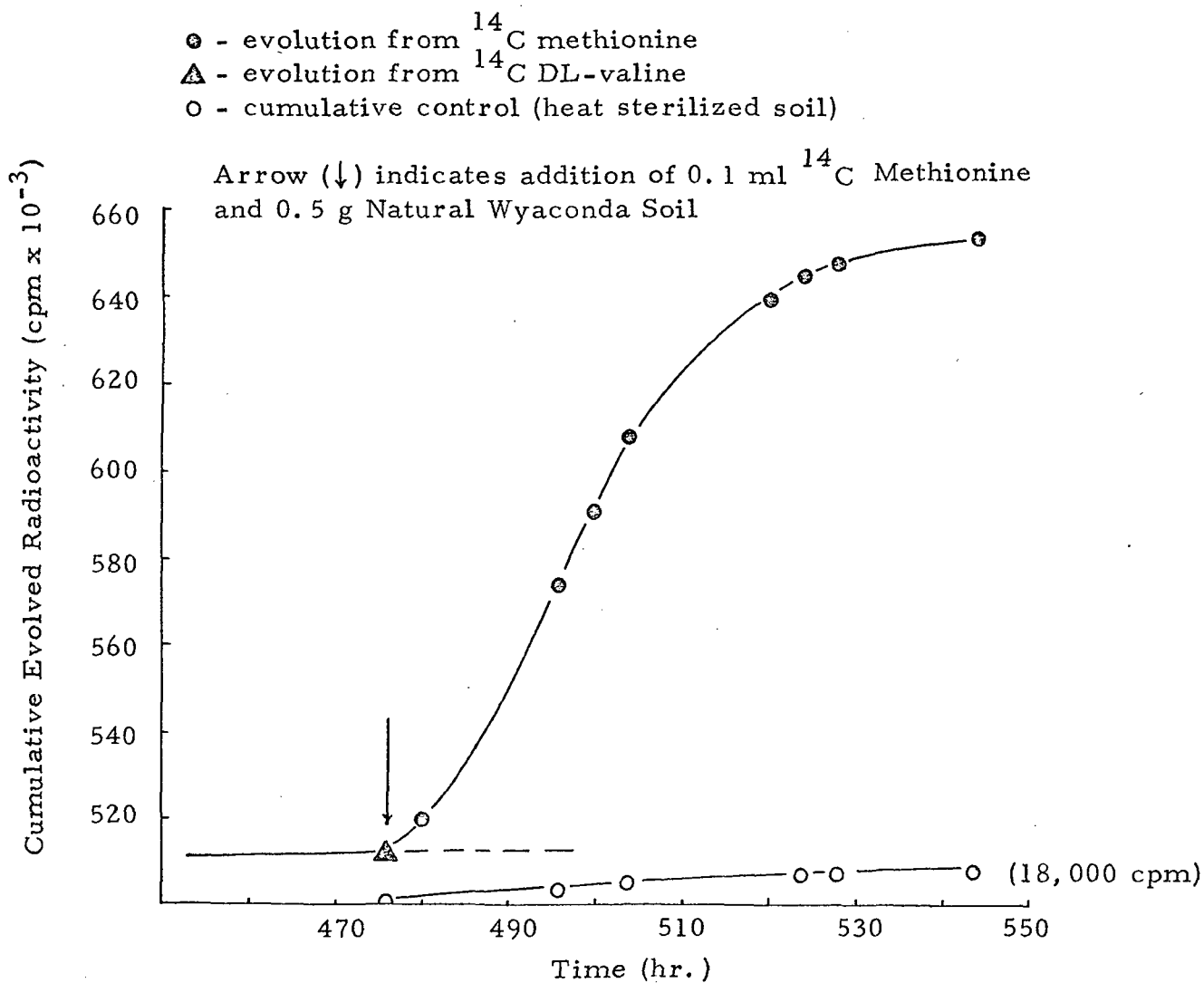
Evolution of $^{14}\text{CO}_2$ from ^{14}C DL-Valine
Multiple Addition Mode



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FIGURE 35

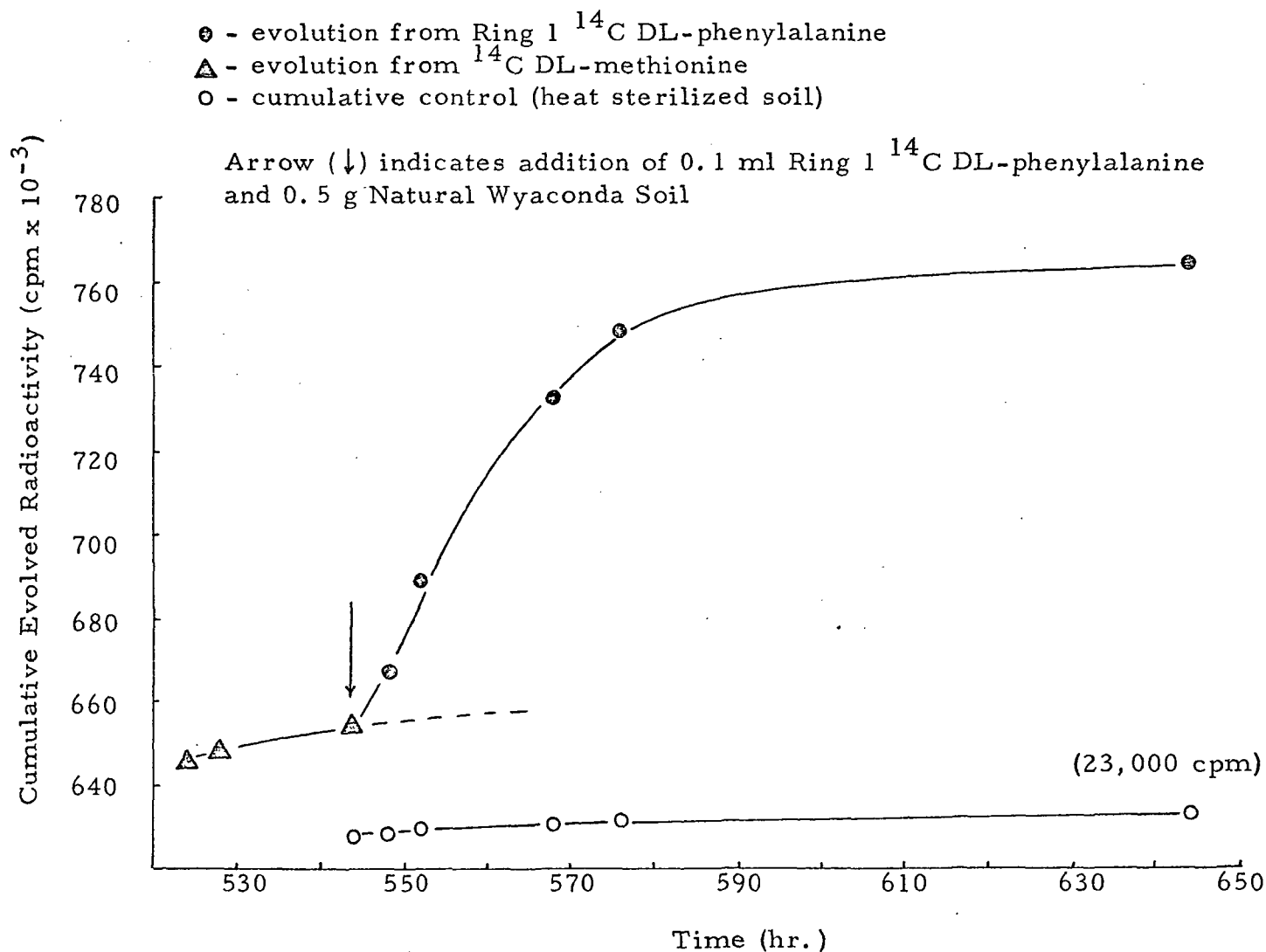
Evolution of $^{14}\text{CO}_2$ from ^{14}C Methionine
Multiple Addition Mode



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FIGURE 36

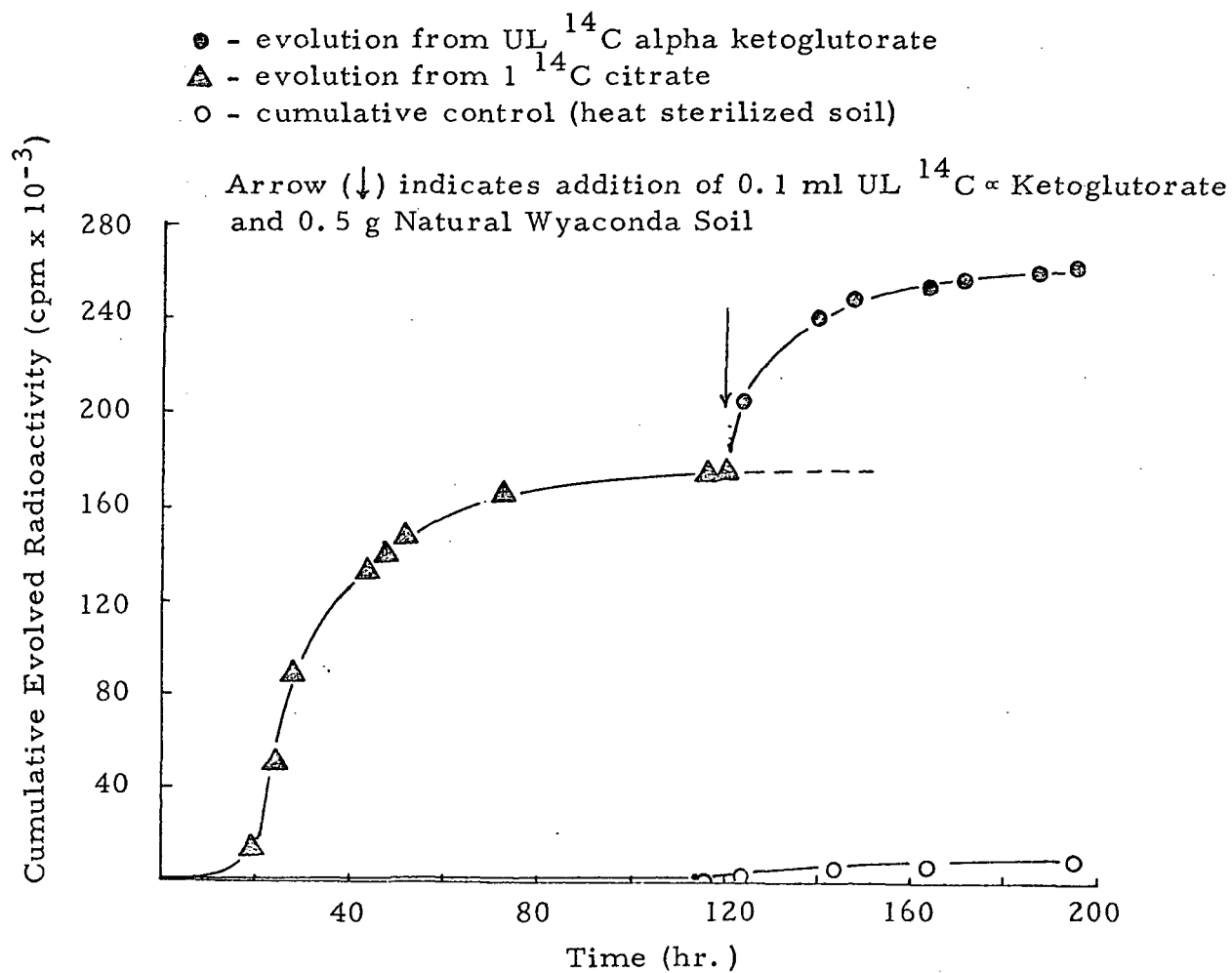
Evolution of $^{14}\text{CO}_2$ from ^{14}C DL-Phenylalanine
Multiple Addition Mode



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FIGURE 37

Evolution of $^{14}\text{CO}_2$ from ^{14}C Citrate and ^{14}C Alpha-Ketoglutarate
Multiple Addition Mode



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TABLE 4
A Comparison of Single Addition, Combination
and Multiple Addition of Substrates
14CO Evolution by Natural Wyaconda Soil

Substrate	Single Addition		Multiple Addition		Combination	
	Cumulative (cpm)	Ratio (Viable/Sterile)	Cumulative (cpm)	Ratio (Viable/Sterile)	Cum.	Ratio
<u>Carbohydrates</u>						
D-glucose	70,000	1,200	73,000	1,100		
L-glucose	20,000	44	30,000	180		
Sucrose	100,000	370	95,000	195		
Lactose	90,000	250	105,000	140		
Xylose	50,000	62	80,000	260		
Cellulose	50,000	68				
Avg.	63,000	150	76,000	145	55,000	65
<u>Amino Acids</u>						
DL-Phenylalanine	60,000	100	105,000	23		
L-valine	60,000	33	140,000	84		
DL-valine	60,000	18	115,000	35		
Methionine	40,000	12	140,000	22		
Glycine	100,000	40	105,000	84		
D-alanine	60,000	100	60,000	101		
L-alanine	60,000	100	83,000	100		
Avg.	63,000	47	93,000	25	60,000	20
<u>Krebs Cycle</u>						
Citrate	120,000	110	170,000	100		
Alpha-Ketoglutarate	80,000	10	85,000	11		
Avg.	100,000	10	127,000	25	95,000	20

*Cumulative viable/cumulative sterile

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for both addition modes during carbohydrate additions. However, the multiple additions of amino acids resulted in a greater cumulative evolved radioactivity than the single additions. It may be that organisms which were conditioned to amino acids by prior additions produced CO_2 more rapidly and efficiently from later additions. Precautions to avoid contact of medium with cells stimulated by an earlier medium addition should be taken. Nevertheless, this experiment demonstrated the feasibility of testing many different substrates with relatively simple equipment, similar to that currently planned for Viking 1975.

D. Inhibitor Study

A series of experiments were conducted to test the feasibility of the multiple addition mode as a means of determining critical inhibitory concentrations of various antimetabolites. The experimental design was to add an inhibitor at a given concentration to ^{14}C -labeled growth medium which was added in turn to natural soil in a culture chamber. Evolved radioactivity was used as the criterion for growth and/or metabolism. Sequential additions of soil medium and a range of inhibitor concentrations were added to a single growth chamber to test the possibility of establishing a soil medium inhibitory concentration with a single culture chamber. The procedure used in these experiments was as follows:

1. Natural Wyaconda and heat-sterilized Wyaconda soil (0.5 g) in duplicate were added to four sterile culture tubes.

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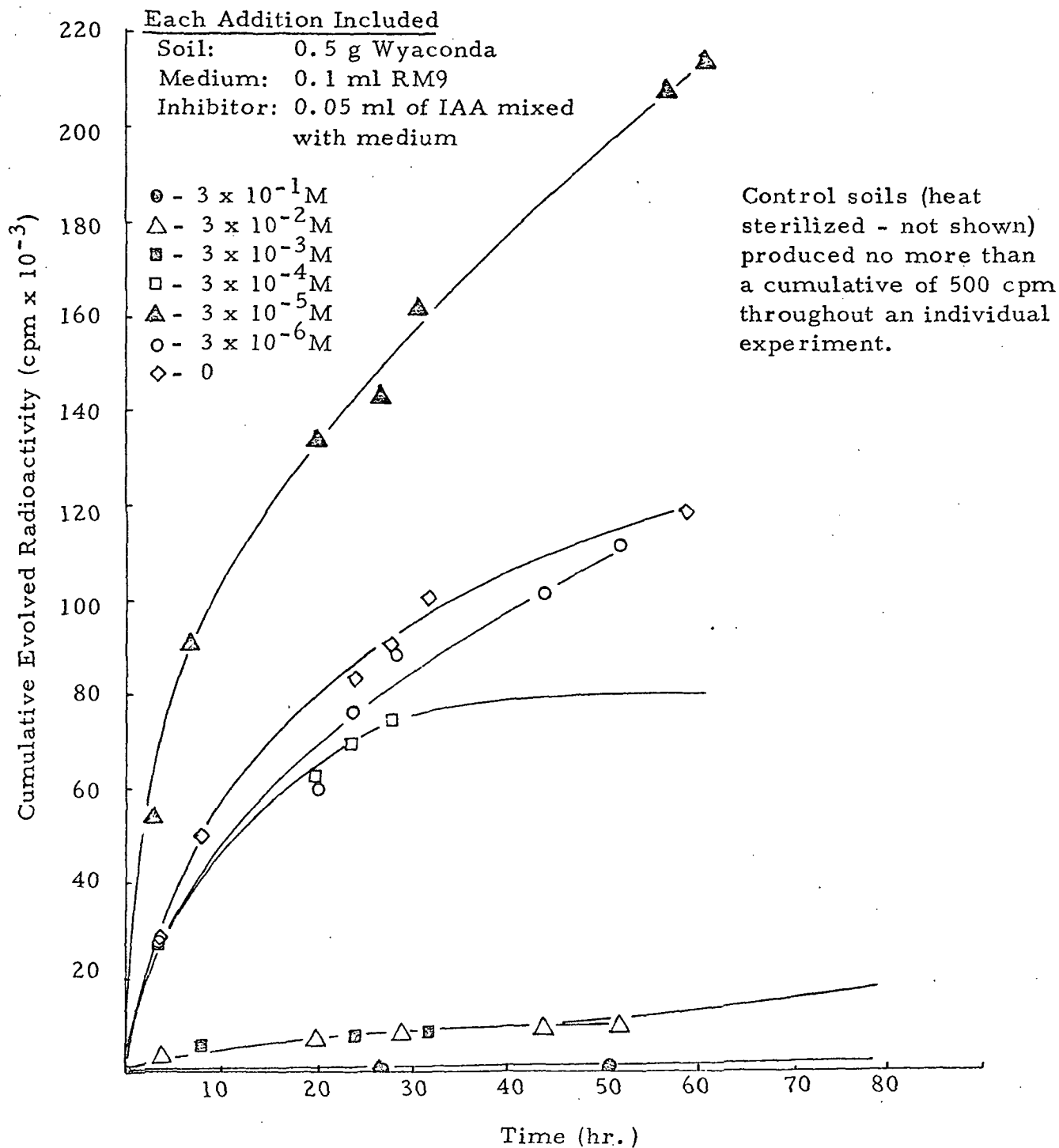
2. RM9 medium containing UL ^{14}C D-glucose $6\text{ }\mu\text{Ci/ml}$, $2.5 \times 10^{-4}\text{M}$; ^{14}C formate $1\text{ }\mu\text{Ci/ml}$, $2.5 \times 10^{-4}\text{M}$; UL ^{14}C glycine, $2\text{ }\mu\text{Ci/ml}$, $2.5 \times 10^{-4}\text{M}$; and UL ^{14}C -lactate $3\text{ }\mu\text{Ci/ml}$, $2.5 \times 10^{-4}\text{M}$ was mixed with a given concentration of iodoacetic acid (IAA) in the ratio of 0.1 ml RM9:0.05 ml (IAA).
3. This mixture (0.15 ml) was added to each of the soils in the culture tubes.
4. Evolved radioactivity was collected with $\text{Ba}(\text{OH})_2$ impregnated filter pads which were changed at intervals.
5. After the evolution of radioactivity had reached an apparent plateau, the addition of soil, medium and a different concentration of IAA was added to the same culture tubes used in the preceding experiment. Control soil (heat sterilized) was always added to the same culture tubes used previously for control soils.

It was expected that the order of addition, i.e., higher to lower concentrations or visa versa, might affect results. The experiment was, therefore, conducted with subsequent additions proceeding both toward a higher concentration of IAA from zero concentration and toward zero concentration from an initial concentration of 10^{-1}M . The results of this experiment are shown in Figures 38a and 38b.

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FIGURE 38a

Inhibition by Iodoacetic Acid (IAA), Multiple
Addition Mode, Concentration Decreasing



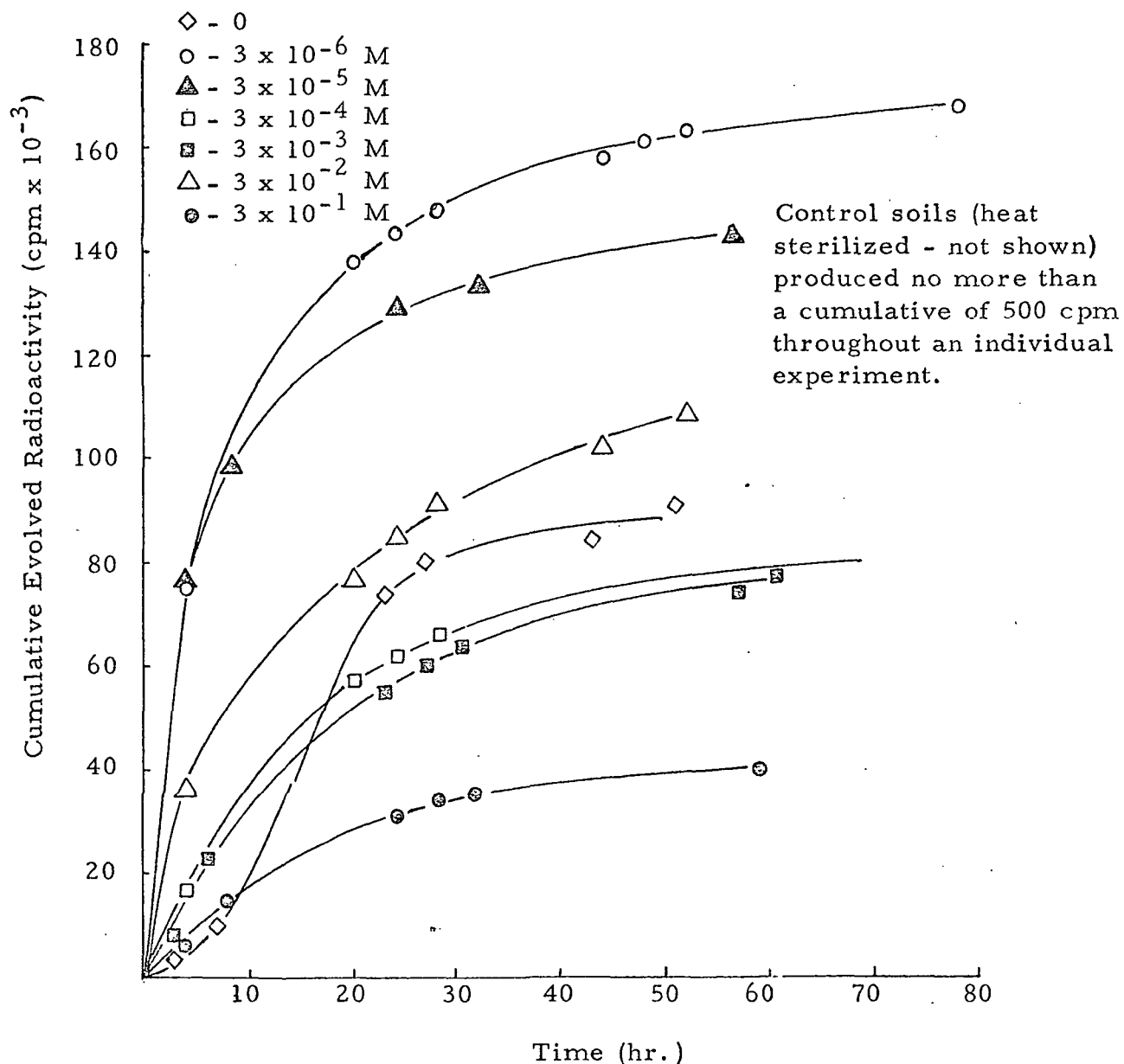
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FIGURE 38b

Inhibition by Iodoacetic Acid (IAA), Multiple
Addition Mode, Concentration Increasing

Each Addition Included:

Soil: 0.5 g Wyaconda
Medium: 0.1 ml RM9
Inhibitor: 0.05 ml of IAA mixed
with medium



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Figures 39-44 show the $^{14}\text{CO}_2$ evolution which resulted from successive increasing concentrations of IAA in ^{14}C labeled medium and soil to a single vial. Incubation time and cumulative radioactive evolution are progressive and continuous in these figures. The plateau in $^{14}\text{CO}_2$ evolution from proceeding soil-medium-inhibitor additions is shown for each subsequent addition. Figures 45a-49 show the results of adding successive decreasing concentrations of inhibitor.

Sequential additions of IAA from a high of 10^{-1}M to lower concentrations, as shown in Figure 38a, gave good inhibition at concentrations of 10^{-1} , 10^{-2} and 10^{-3}M . The 10^{-4}M concentration showed only slight inhibition. There may have been stimulation at the 10^{-5}M concentration.

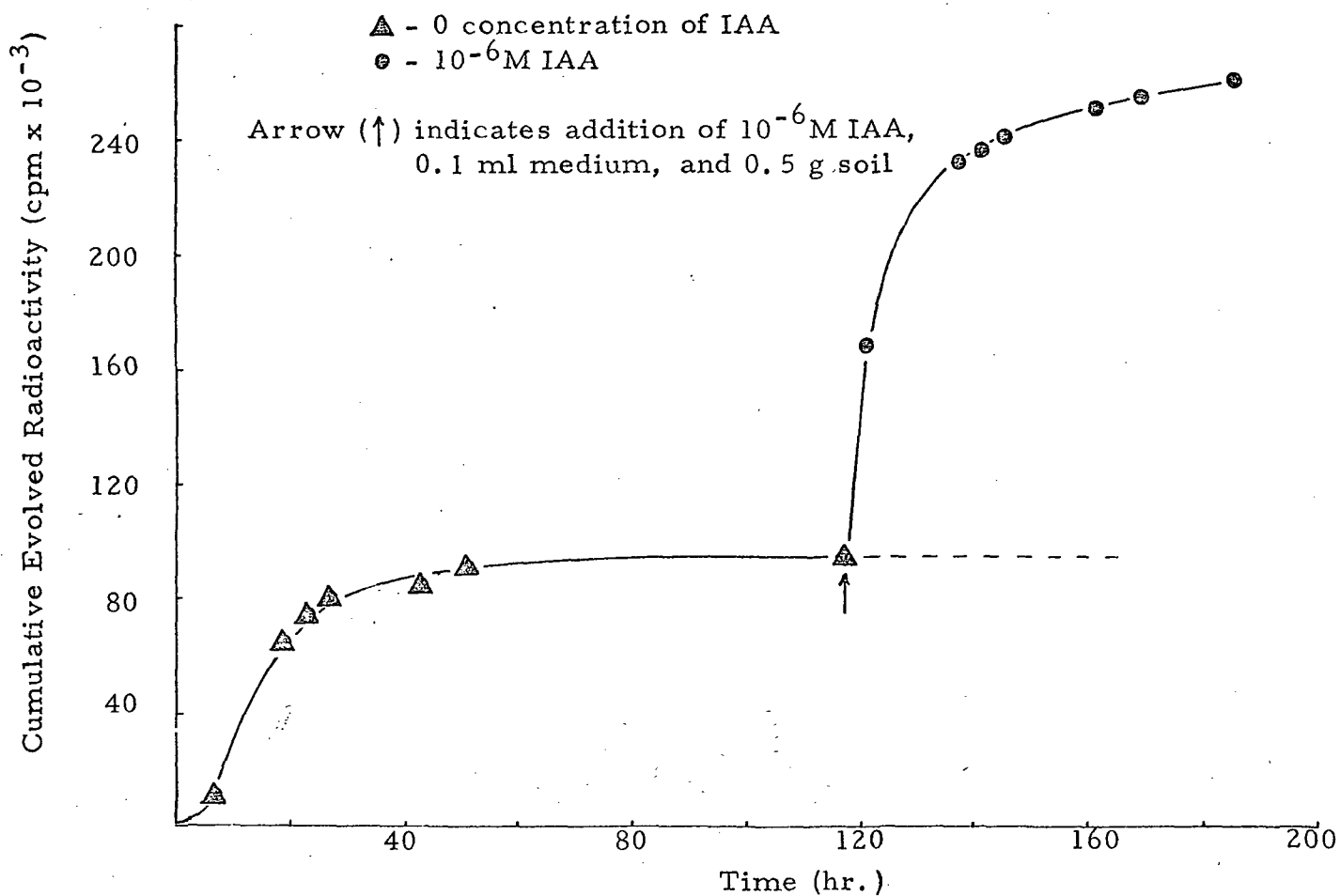
Sequential additions of IAA from zero to higher concentrations produced the results as shown in Figure 38b. The initial addition, containing no IAA produced an "S" shaped curve which differed from all other curves by showing a lag in evolution. These results are in agreement with those shown in Figure 6, which were obtained earlier. These results mean that sufficient liquid was present to percolate down through the soil and contact acclimated cells produced by the previous and underlying soil and medium. The gradual increase in concentrations of IAA probably resulted in the growth of resistant types or mutants since even the highest concentration of IAA tested (10^{-1}M) failed to produce much more than 50% inhibition of the cumulative response obtained when no IAA was present. As in the

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FIGURE 39

Inhibition by Iodoacetic Acid (IAA), Multiple
Addition Mode, Increasing Concentration

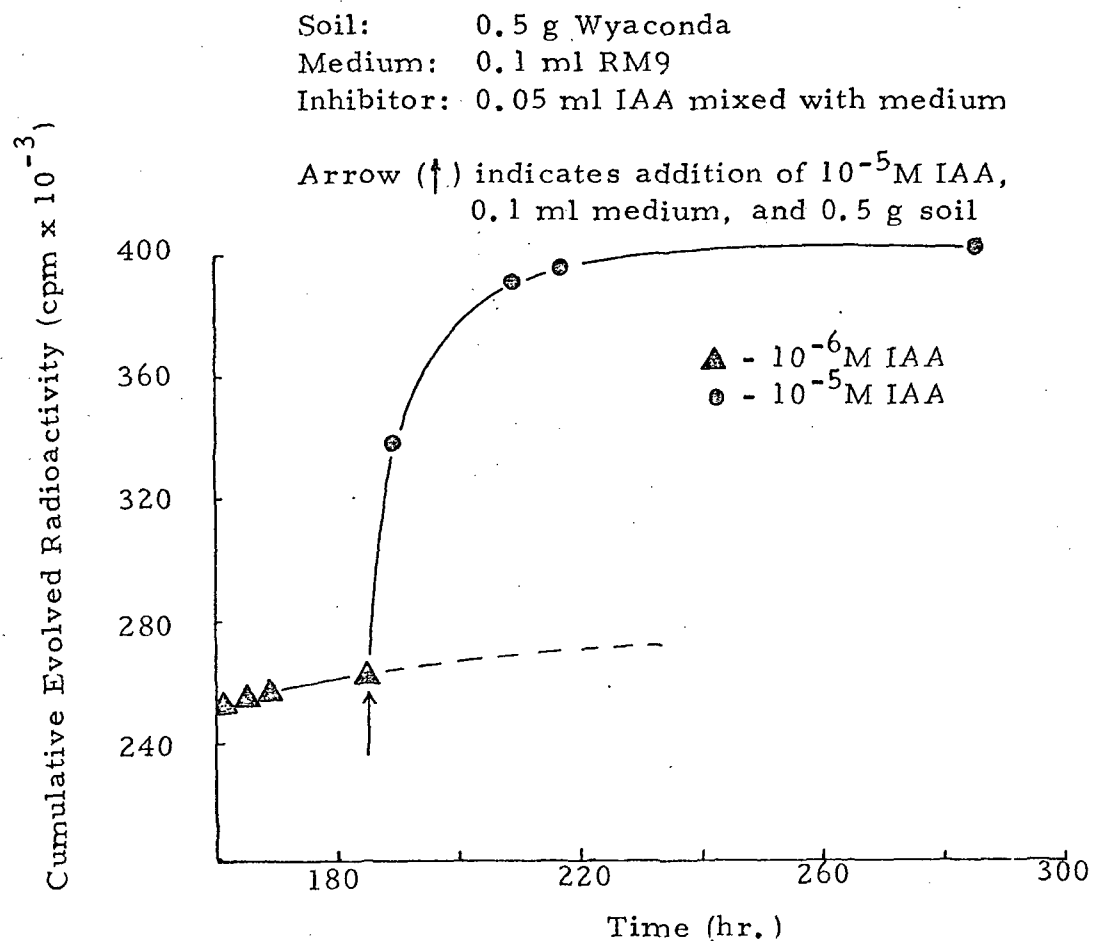
Soil: 0.5 g Wyaconda
Medium: 0.1 ml RM9
Inhibitor: 0.05 ml IAA mixed with medium



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FIGURE 40

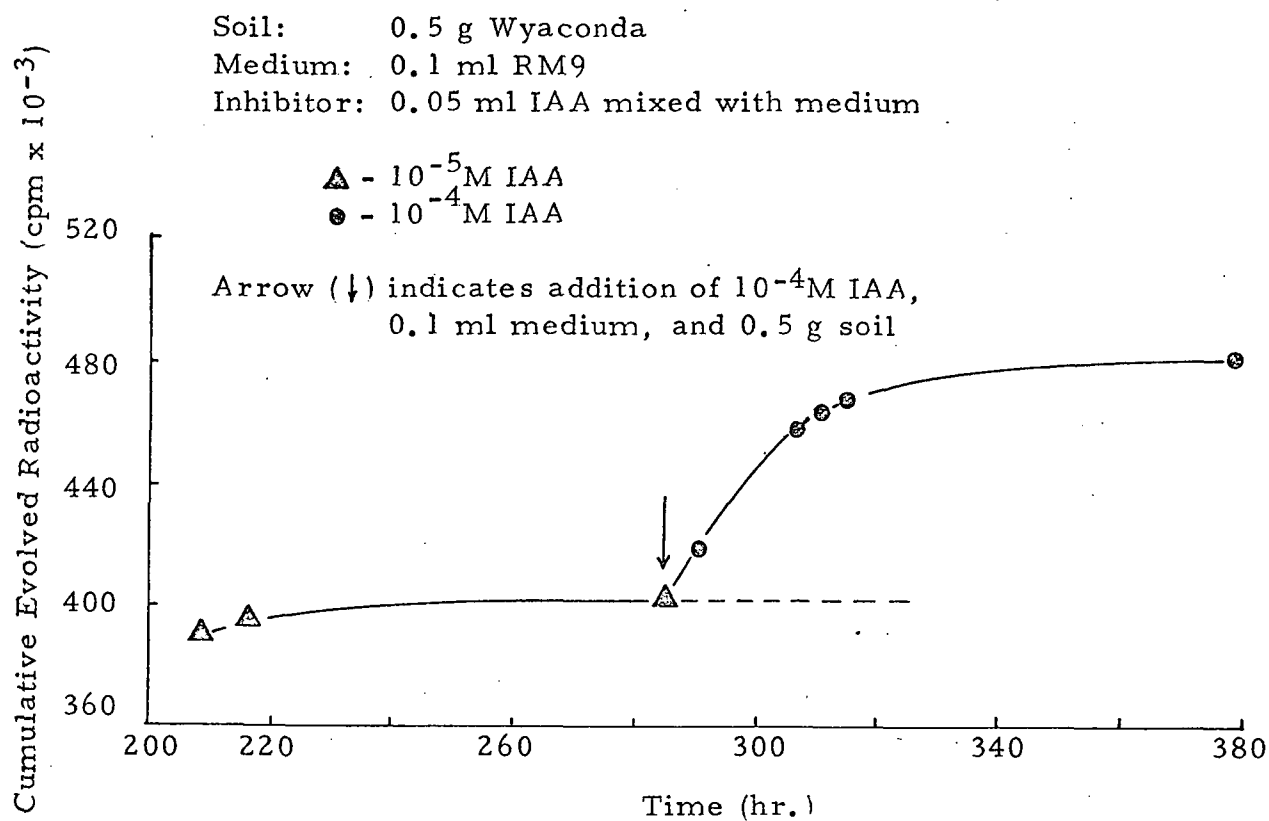
Inhibition by Iodoacetic Acid (IAA), Multiple
Addition Mode, Increasing Concentration



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FIGURE 41

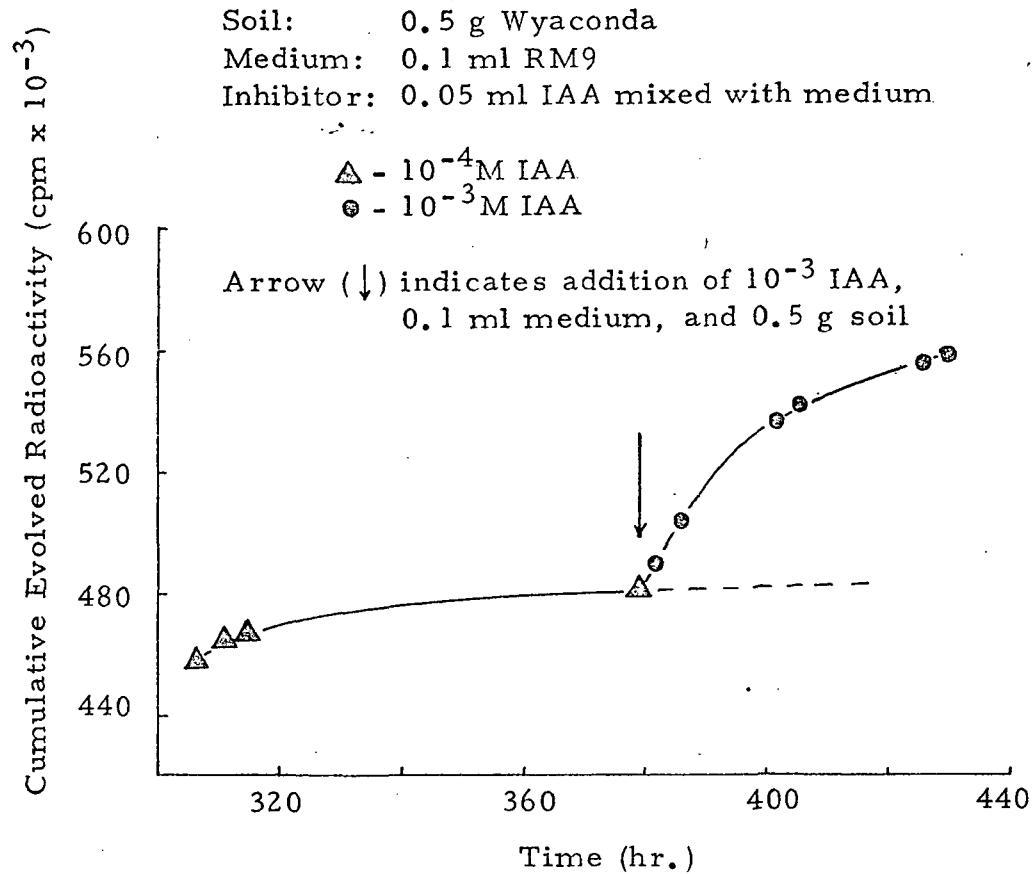
Inhibition by Iodoacetic Acid (IAA), Multiple
Addition Mode, Increasing Concentration



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FIGURE 42

Inhibition by Iodoacetic Acid (IAA), Multiple
Addition Mode, Increasing Concentration



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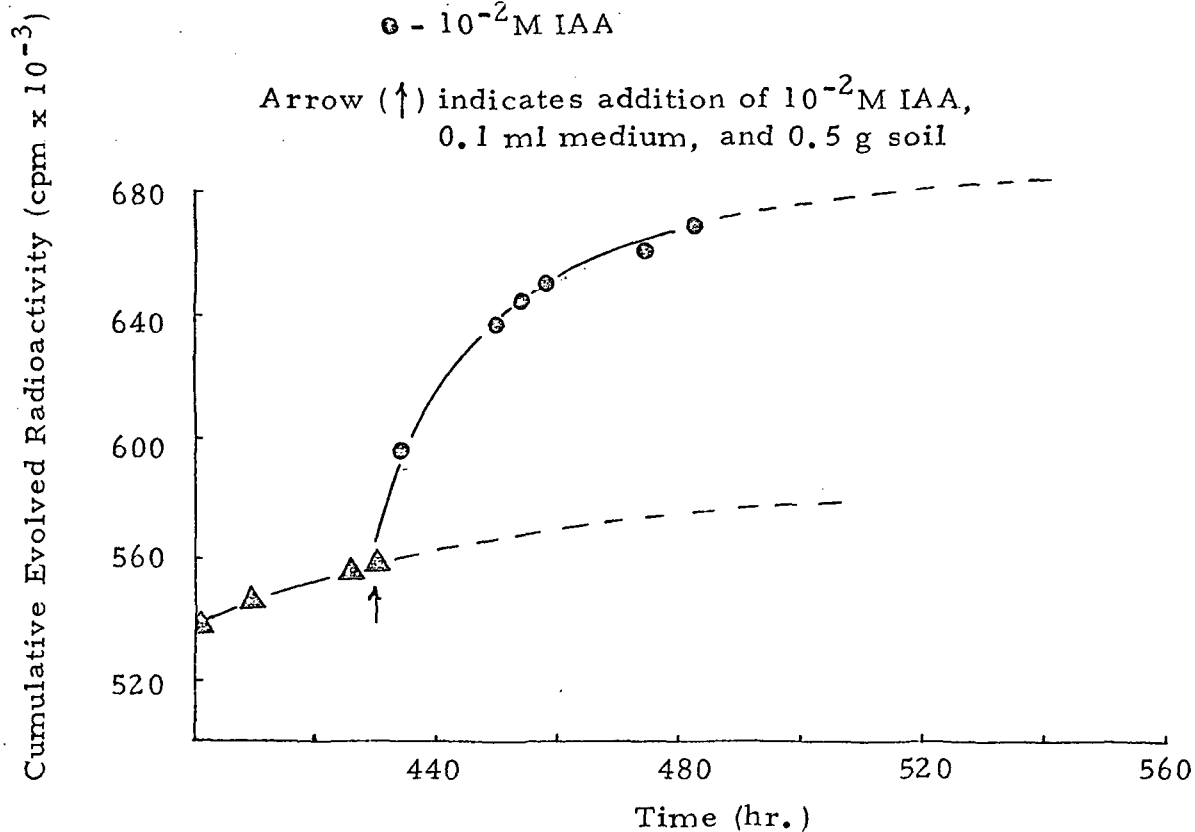
FIGURE 43

Inhibition by Iodoacetic Acid (IAA), Multiple
Addition Mode, Increasing Concentration

Soil: 0.5 g Wyaconda
Medium: 0.1 ml RM9
Inhibitor: 0.05 ml IAA mixed with medium

△ - 10^{-3} M IAA
● - 10^{-2} M IAA

Arrow (↑) indicates addition of 10^{-2} M IAA,
0.1 ml medium, and 0.5 g soil

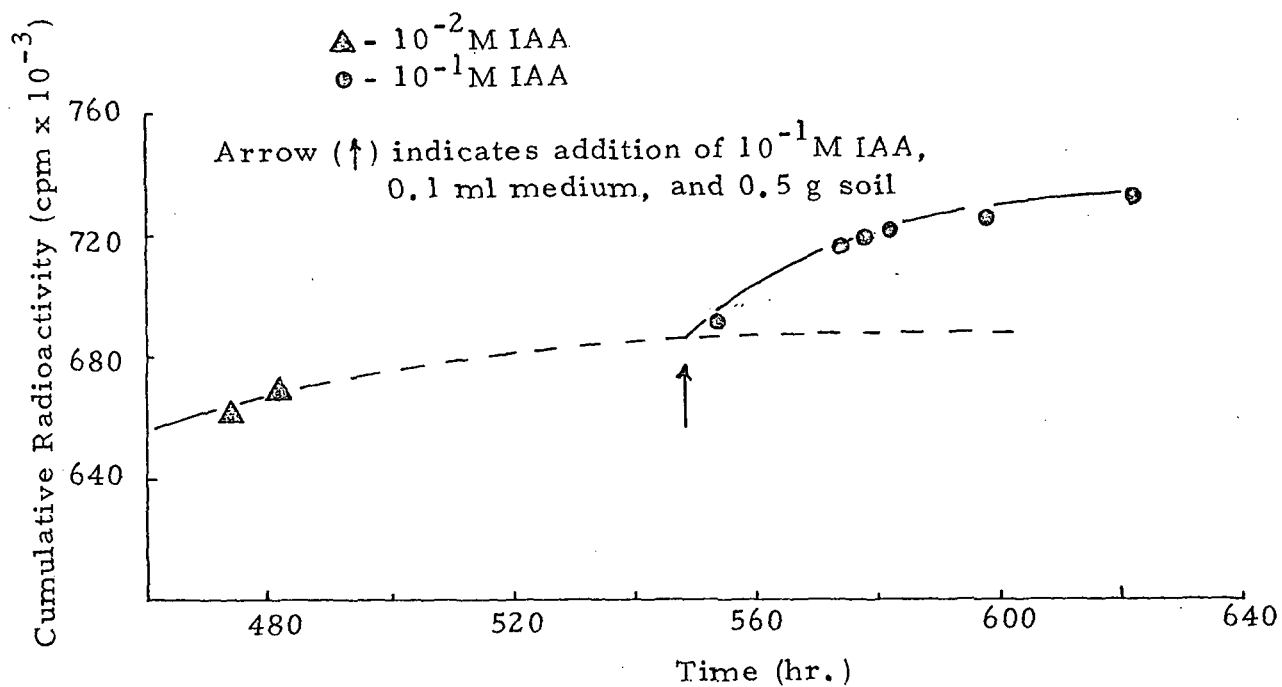


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FIGURE 44

Inhibition by Iodoacetic Acid (IAA), Multiple
Addition Mode, Increasing Concentration

Soil: 0.5 g Wyaconda
Medium: 0.1 ml RM9
Inhibitor: 0.05 ml IAA mixed with medium



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FIGURE 45a

Inhibition by Iodoacetic Acid (IAA) Multiple
 Addition Mode, Decreasing Concentration

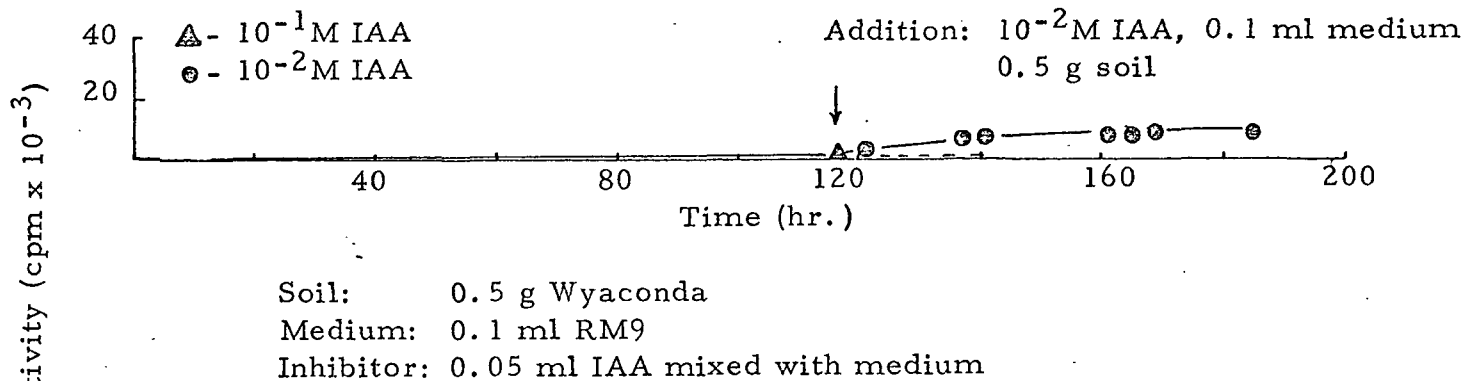
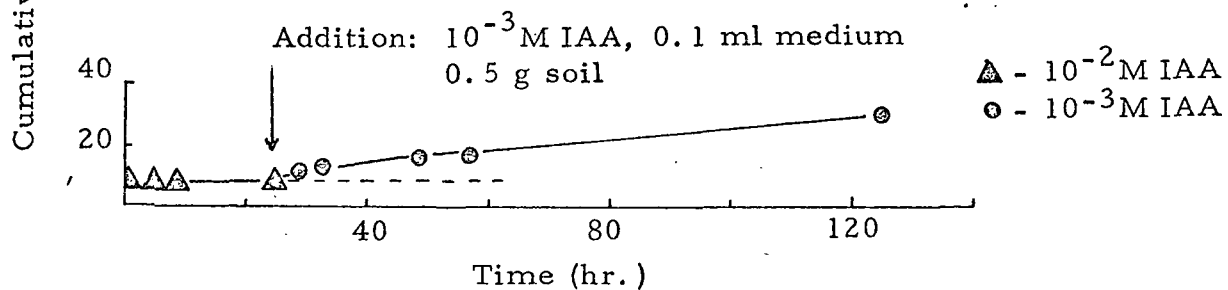


FIGURE 45b

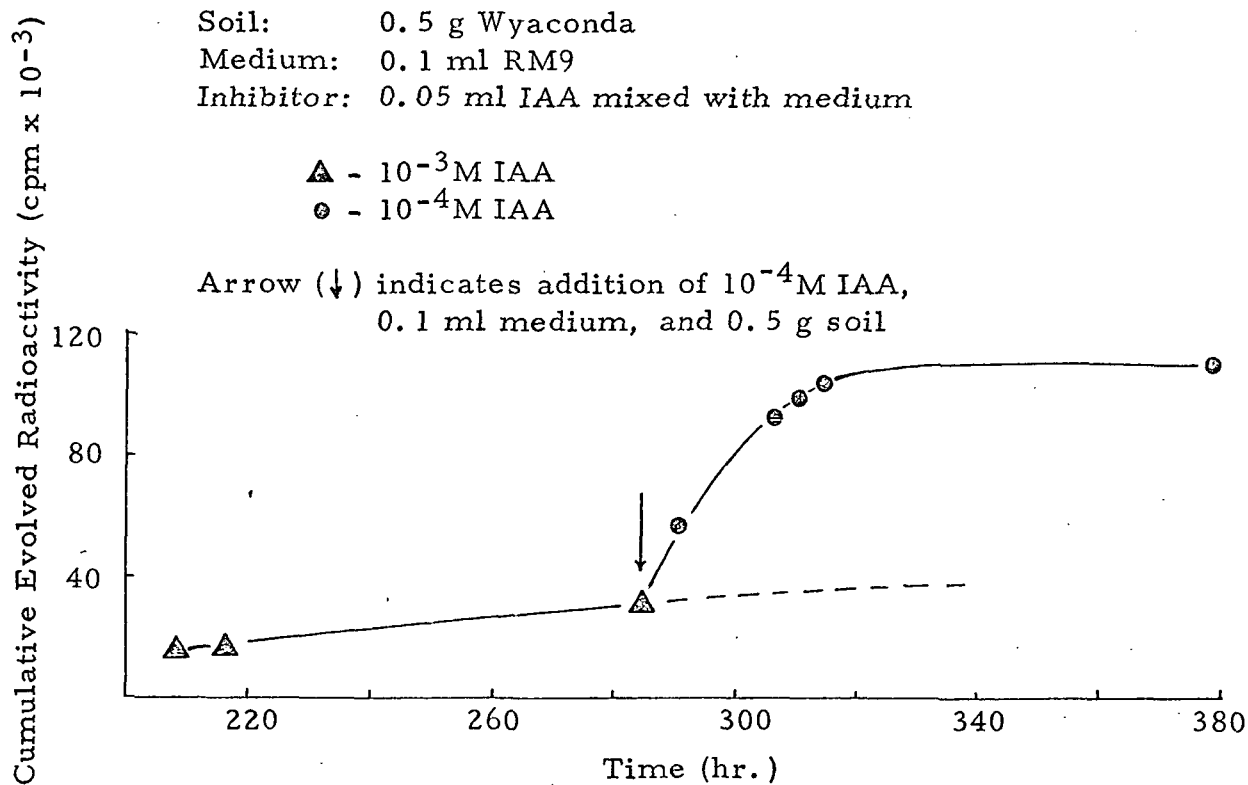
Inhibition of Iodoacetic Acid (IAA), Multiple
 Addition Mode, Decreasing Concentration



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FIGURE 46

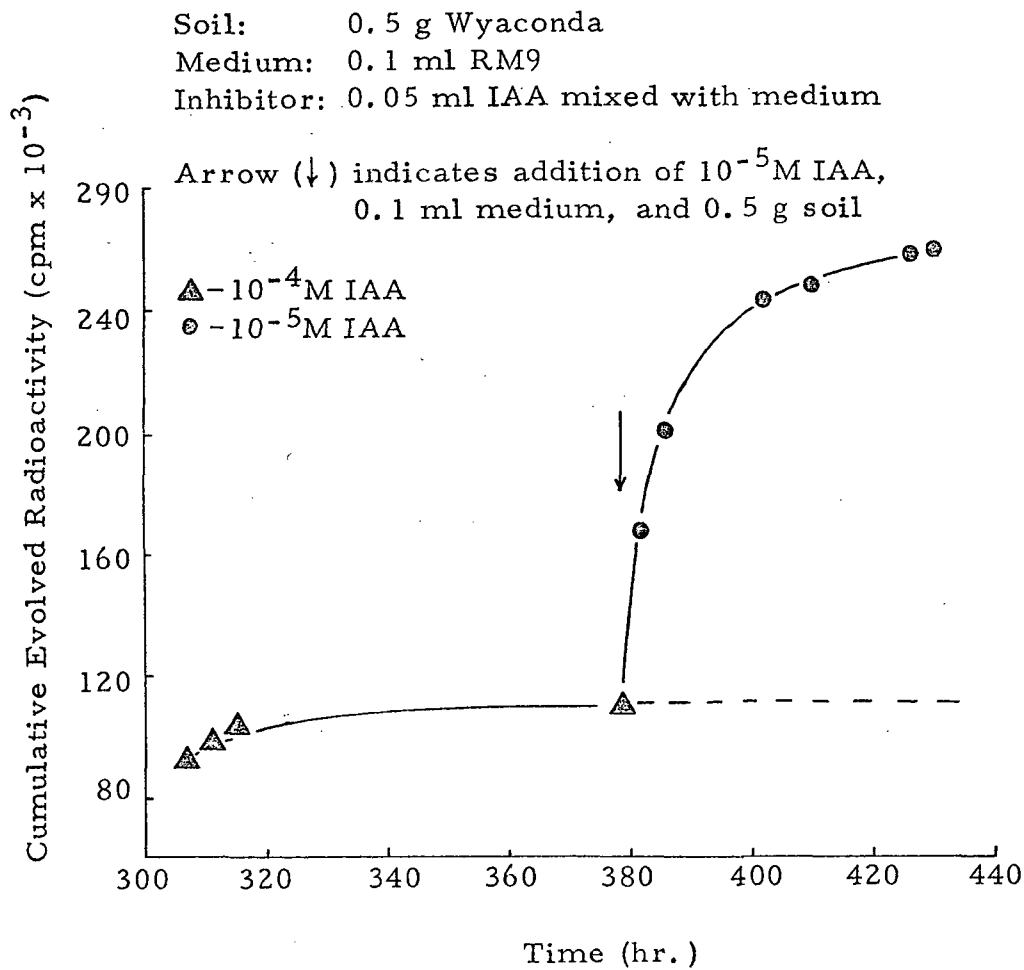
Inhibition by Iodoacetic Acid (IAA), Multiple
Addition Mode, Decreasing Concentration



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FIGURE 47

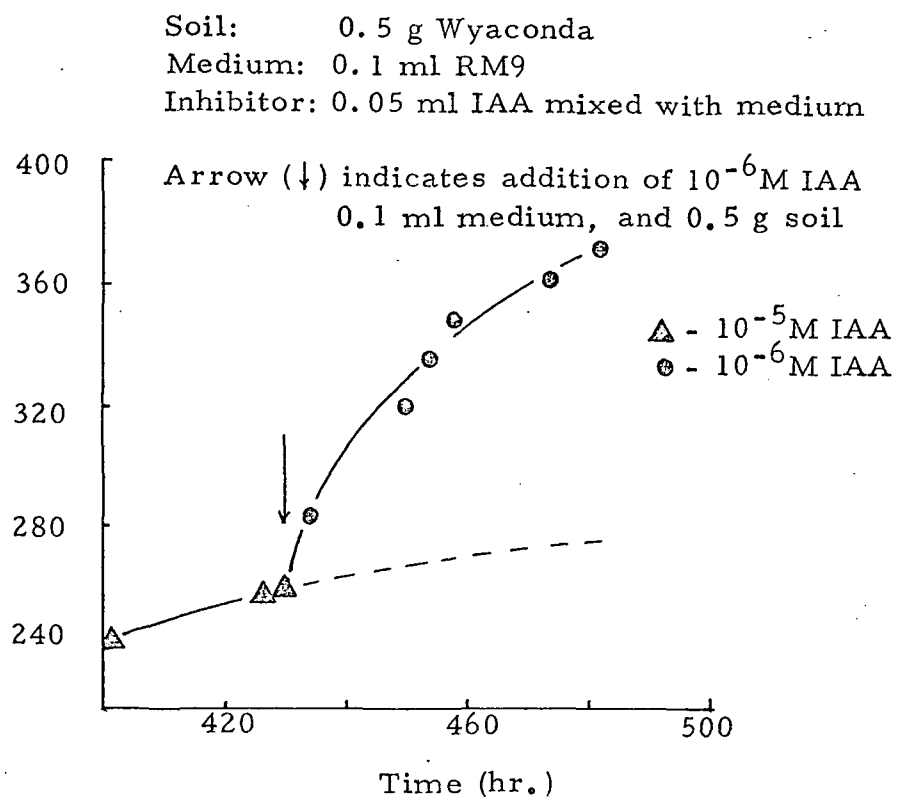
Inhibition by Iodoacetic Acid (IAA), Multiple
Addition Mode, Decreasing Concentration



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FIGURE 48

Inhibition by Iodoacetic Acid (IAA), Multiple
Addition Mode, Decreasing Concentration

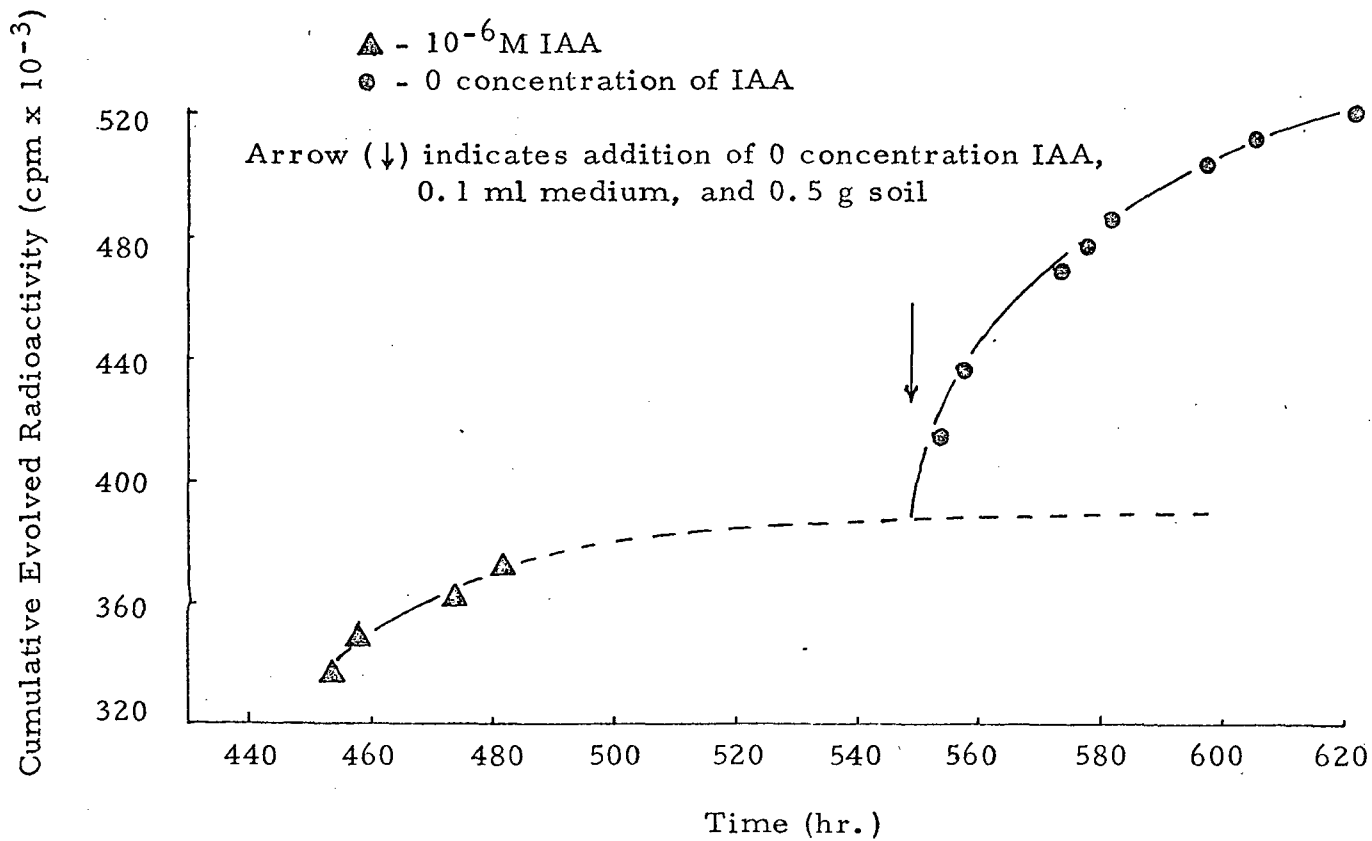


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FIGURE 49

Inhibition by Iodoacetic Acid (IAA), Multiple
Addition Mode, Decreasing Concentration

Soil: 0.5 g Wyaconda
Medium: 0.1 ml RM 9
Inhibitor: 0.05 ml IAA mixed with medium



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previous experiment, there appeared to be stimulation of ^{14}C evolution by low concentrations of IAA.

In an attempt to verify the results of the multiple addition experiment, a single addition experiment was performed in which each concentration of IAA was added to soil in separate planchets. The results of this experiment, which are shown in Figure 50, support the multiple addition experiment when addition was in order of decreasing concentration. However, less inhibition at 10^{-3}M IAA was observed in the single addition experiment. The actual concentration in the multiple addition experiments was probably much higher due to the movement upward of IAA from underlying experiments conducted with 10^{-2} and 10^{-1}M concentrations.

The values graphed in Figure 50 are the averages of duplicates which showed considerable spread at the low and zero concentrations of IAA. Nevertheless, it appears that 10^{-6}M IAA may have been stimulatory.

This series of experiments demonstrates the possible utility of the multiple addition mode. However, the influence of an underlying soil culture was observed. Further tests should minimize this influence by either using a larger soil sample so that the new addition of medium does not reach the underlying acclimated cells or using a barrier of inert material.

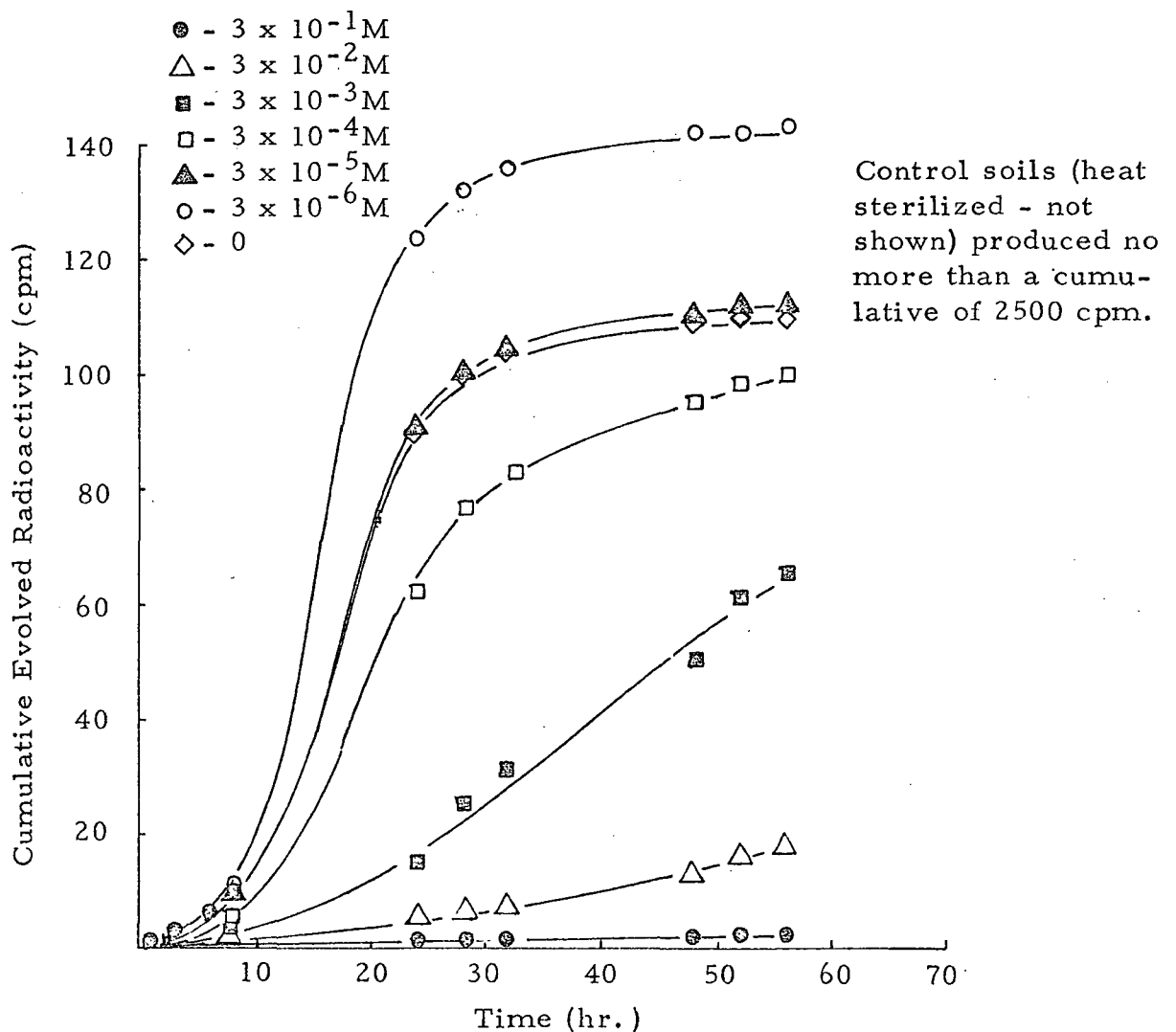
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FIGURE 50

Inhibition by Iodoacetic Acid (IAA), Single
Addition Mode

Experimental Conditions

Soil: 0.5 g Wyaconda
Medium: 0.1 ml RM9
Inhibitor: 0.05 ml IAA mixed
with medium



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II. NITROGEN FIXATION

A. Introduction

Considerable progress in the understanding of nitrogen fixing systems has been made in recent years. In 1966, Scholhorn and Burris(21), and Dilworth, J. J. (2) demonstrated that the nitrogen fixing complex reduces acetylene to ethylene. This finding led the way to the development of simplified techniques for cell free studies (2, 4, 5) and in situ analysis of natural soil and water populations (6, 7, 3, 5).

The possibility of developing the ethylene reduction assay for incorporation into the complement of independent life detection experiments currently being developed in the AMML program appears attractive from several standpoints: 1.) No life detection system, thus far considered, would monitor nitrogen metabolism. Any finding concerning the presence or absence of nitrogen fixation would provide a significant contribution in knowledge. 2.) The technique is highly sensitive (two to three cells produce sufficient C_2H_4 for detection (3). 3.) There is no evidence of nonbiological ethylene production. Production of ethylene by non- N_2 -fixing organisms is less than 0.1% of that obtained from N_2 -grown cultures of Azotobacter vinelandii or Clostridium pasteurianum. 4.) Proven in situ capabilities of the assay indicate the test is well suited for soil

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analysis. 5.) Soil samples analyzed during our study show much enhanced ethylene production in the light. The possibility of this procedure doubling as a sensitive monitor of phototrophic activity as well as nitrogen fixation makes it especially desirable for the AMML. 6.) Instrumentation technology has already been established. The basic technique is very simple.

1. Biochemistry of Nitrogen Fixation

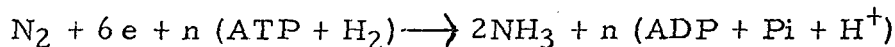
Since 1960, the understanding of nitrogen fixation has advanced tremendously. For reviews of the present state of knowledge, the reader is referred to Fottrell, 1968 (4), Postgate, 1970 (9), and Hardy, et. al., 1970 (3).

Two protein fractions of the nitrogenase system in both anaerobic and aerobic organisms have been identified and named molybdoferredoxin and azoferredoxin. The former contains molybdenum and nonhaem iron and is reasonably stable to air. The azoferredoxin possesses two iron and two labile sulphide groups and is irreversibly damaged by oxygen even if isolated from an aerobic microbe. Both fractions are required for nitrogen fixation or acetylene reduction.

Also required for N_2 reduction is ATP and an electron donor. Pyruvate has been shown to function as both electron donor and energy source wherein the phosphoroclastic reaction forms acetyl phosphate which gives rise to ATP. Inorganic electron donors such as dithionite and potassium borohydride have also been used in the presence of an ATP

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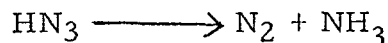
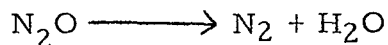
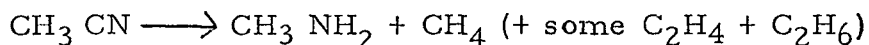
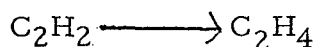
generating system. The system also has a requirement for a divalent-cation. Mg^{++} , Mn^{++} , Co^{++} , Fe^{++} and Ni^{++} have all been shown to function in that order of effectiveness. N_2 is the electron acceptor in the nitrogen fixing scheme. Therefore, the reduction of N_2 by the nitrogenase system is shown by the following reaction:



In addition, H^+ is reduced to H_2 . This reaction occurs even in the absence of N_2 reduction. Carbon monoxide competitively inhibits N_2 reduction, but not the hydrolysis of ATP and reduction of H^+ . Of specific interest is the fact that several other electron acceptors can function with the N_2 ase system. These compounds and their reduction products are shown in Table 5.

TABLE 5

Substrates Reduced by Nitrogenase



Aside from the interesting fact that nitrogenase can couple with these various substrates, the reduction of ethylene has provided a convenient, simple and highly sensitive method of monitoring the rate of nitrogen

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fixing metabolism. Results of the method have been compared favorably to other conventional techniques of measuring N_2 fixation. However, the sensitivity and precision of ethylene measurement by gas chromatography greatly exceeds that achieved in Kjeldahl determinations, and is much easier to perform and less expensive than ^{15}N procedures. Techniques for in situ studies of soil microbes and symbiotic associations based on acetylene reduction have greatly expanded knowledge concerning the significance and distribution of nitrogen fixing organisms.

2. Microorganisms Conducting Nitrogen Fixation

Within aerobic microbes, the phenomenon of nitrogen fixation is somewhat restricted. Blue-green algae, members of the family Azotobacteriaceae and Mycobacterium flavium, are the only known aerobic organisms which fix nitrogen. However, the process is relatively widespread among facultative and obligate anaerobes. Findings concerning the inhibitory effects of oxygen on Azotobacter N_2 ase systems (11) and the specialized compartmentation which has developed in blue-green algae (heterocysts appear to be the site of N_2 ase activity) tends to indicate that the process is largely anaerobic. Thus, our terrestrial knowledge supports the use of the method as a life detection test on Mars.

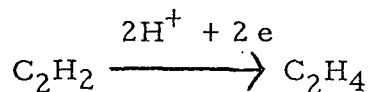
Nitrogen fixation of agronomic importance appears to be limited to symbiotic associations (3); however, these systems are not fully understood. At least some cases of N_2 fixation in root modules have been found to be localized in the associated bacteroids.

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3. Sensitivity, Inhibition and False Positives

Measurement of ethylene is possible at levels as low as approximately 10^{-12} moles in a 200 μ l sample. Hardy, et. al., have determined that Azotobacter produces approximately 10^{-12} moles C_2H_4 /hr./cell (3). Therefore, the sensitivity of the assay is in the order of only a few cells. Stewart, et. al. (12) have tested various pure cultures of blue-green algae and find that acetylene reduction in some cultures occurs at the rate of 2.5×10^{-10} moles/ μ g of protein/hr. Drozd and Postgate (11) studying conditions of optimum PO_2 in cultures of Azotobacter, have found acetylene reduction rates of 2.4×10^{-9} moles/ μ g of protein/hr. This means that approximately ten nonproliferating cells of Azotobacter could be detected in about ten hours.

The stoichiometry of C_2H_2 reduction shows that the number of moles of C_2H_2 reduced is equal to the number of moles of C_2H_4 formed. For each C_2H_2 reduction, two electrons are required:



On the other hand, reduction of N_2 to NH_3 requires the transfer of six electrons. This means that a similar rate of nitrogen fixation would produce one-third more measurable C_2H_4 than NH_3 .

The reduction of C_2H_2 competitively inhibits N_2 reduction, however, in the absence of N_2 , C_2H_4 is the only product. No detectable amounts of C_2H_6 or CH_4 are formed. The rate of N_2 ase activity is similar in the

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presence of either C_2H_2 or N_2 . However, the ATP dependent H_2 -evolving activity of N_2 ase is reduced somewhat in the presence of C_2H_2 . Carbon monoxide which (competitively) inhibits both C_2H_2 or N_2 reduction does not inhibit the evolution of H_2 . The hypothesis of a two-site reduction, one for H^+ and one for all other electron acceptors has, therefore, been proposed. Both of these sites would compete for electrons.

Nitrogenase activity is not displayed by organisms which have been grown on a medium containing fixed nitrogen. The enzyme is either induced or a repressor is removed in the absence of fixed nitrogen. It has also been found that oxygen has an inhibitory effect on ethylene production by whole cells of Azotobacter. Partial pressures of oxygen, considerably below atmospheric, caused significant increases in activity unless the cells were acclimatized by prior culturing techniques.

The saturation constant (K_m) of acetylene for both Clostridium and Azotobacter N_2 ase systems is approximately 0.025 to 0.1 atmosphere (3). Even 0.5 atmosphere is not inhibitory and may be stimulatory to N_2 -grown Azotobacter. However, Rice and Paul, 1971 (7) have recently found that incubation of C. pasteurianum in the presence of 0.005 atm of C_2H_2 resulted in a much greater production of cells than 0.1 atm. They also noted the cells had become pleomorphic and larger than normal under conditions of 0.1 atm C_2H_2 incubation. This problem would only occur under conditions where growth in connection with acetylene reduction was being measured.

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Likewise, cautions concerning the significance of long-term incubation periods have been raised by Hardy* because the release of NH_3 into the medium by growing cells may cause repression of the nitrogenase enzyme.

The possibilities of false positives; i.e., nonbiological reduction of acetylene by soils has been investigated indirectly. Most in situ studies measuring ethylene have included controls to determine the increase in Kjeldahl nitrogen and ^{14}N incorporation. Although the acetylene reduction method is more sensitive, production of ethylene is supported by N_2 fixation measured by the other techniques. Furthermore, ethylene is not produced in the absence of acetylene.

The high degree of sensitivity of the method has also determined that several organisms, which were previously thought to be nitrogen fixers, do not fix nitrogen.

In the present study, heat killed cultures of Azotobacter showed an initial level of N_2 ase activity as measured by acetylene reduction. Further studies are necessary to confirm and characterize this phenomenon.

4. Method for In Situ Acetylene Reduction Studies

Methods for studying the rate of N_2 ase activity or determining the level of nitrogen fixing biomass in natural soils and waters have been developed (3, 7, 6, 12, 13). They consist, quite simply, of

* Personnel Communication

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placing a soil sample in a container which is then flushed with 80% Ar: 20% O₂ (100% Ar or He for anaerobic systems). A quantity of C₂H₂ is injected into the container. After incubation, a sample of gas is removed and assayed by gas chromatography using a flame ionization detector. Quantitation is determined by comparison of the ethylene peak height with a standard curve.

B. Experimental Biology

A series of preliminary experiments have been performed. The results are highly encouraging and demonstrate that the technique is operationally simple, highly sensitive and suitable for automation.

1. Quantitation of Ethylene

An ethylene standard curve was prepared using a series of 68.7 ml serum bottles which were cleaned, dried and capped with rubber serum stoppers. The bottles were then serially diluted, as shown in Table 6, using a 10 ml Hamilton gas-tight syringe.

TABLE 6

Concentrations of Ethylene Used for Standard Curve

<u>Bottle No.</u>	<u>Moles of Ethylene/ml</u>
1	4.06×10^{-6}
2	3.69×10^{-7}
3	3.35×10^{-8}
4	3.05×10^{-9}
5	2.77×10^{-10}
6	2.52×10^{-11}
7	2.29×10^{-12}

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One-half ml samples of the ethylene dilutions shown in Table 6 were injected into a Hewlett Packard 7620A Research Chromatograph (flame ionization detector) fitted with a 9 ft. long, 1/8 in. diameter column containing Porapak-R. Temperature settings were: oven 55°C, injector 80°C, and detector 65°C. The carrier gas was N₂, set at a flow rate of 25 cc/min. A graph of the peak height vs. the moles of ethylene injected is shown in Figure 51. The loss of linearity at low concentrations of ethylene led to the finding that ethylene is retained by the gas-tight syringe. The standard curve was repeated using disposable plastic syringes to make the ethylene dilutions (a separate syringe for each transfer). This second curve, shown in Figure 52, was linear down to the limits of sensitivity of the instrument.

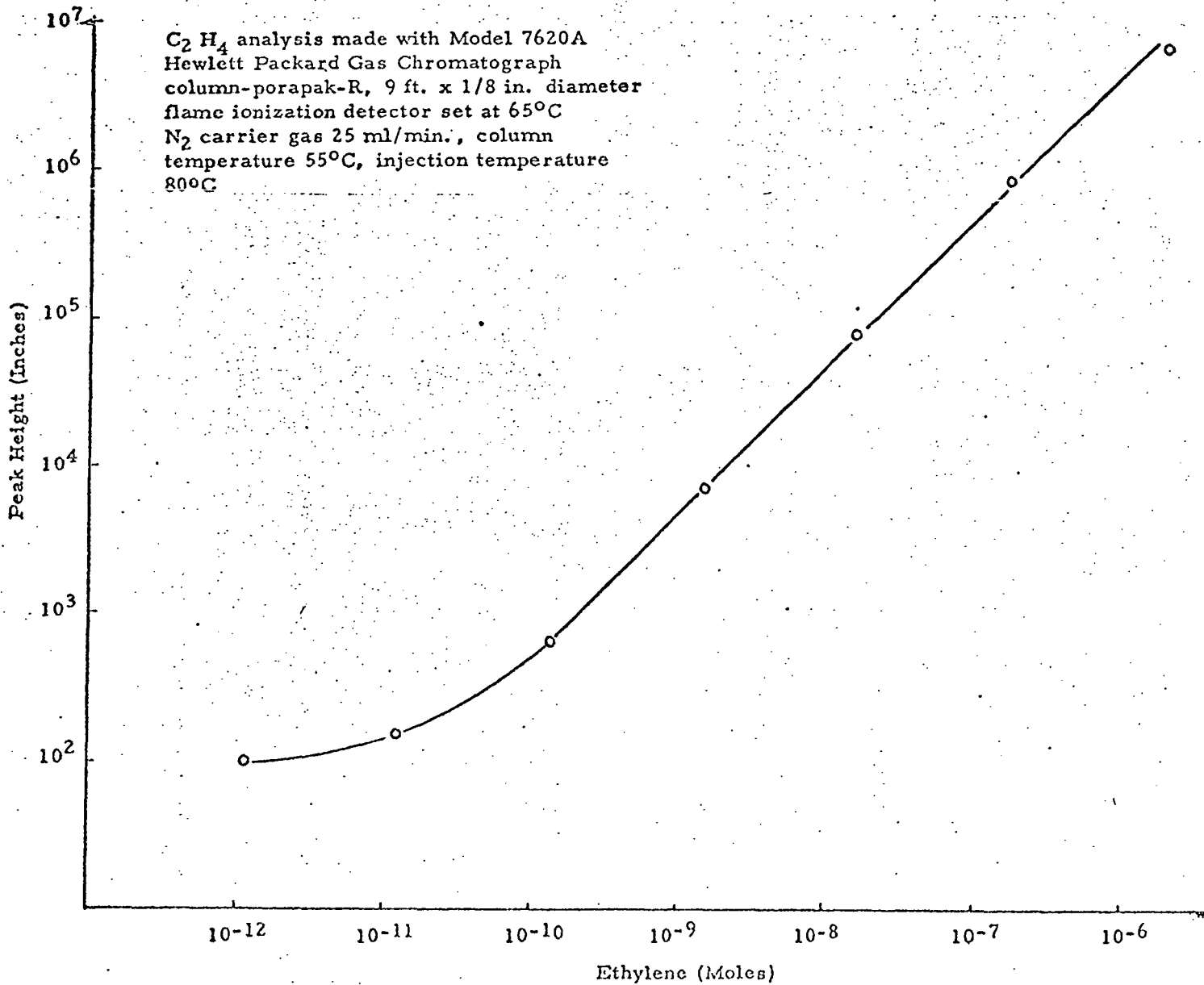
2. Methods of Soil Analysis

Weighed quantities of soil were placed in 3.5 ml serum capped vials which were twice evacuated and flushed for 1.5 min. with a flush gas containing 20.2% O₂ and 475 ppm CO₂ in argon. Afterwards, acetylene was injected into the vials and they were incubated as described in the various experiments. After incubation, 0.5 ml samples of gas were withdrawn from the test vials and injected into the chromatographic apparatus (column and temperature as described for ethylene standard curve).

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FIGURE 51

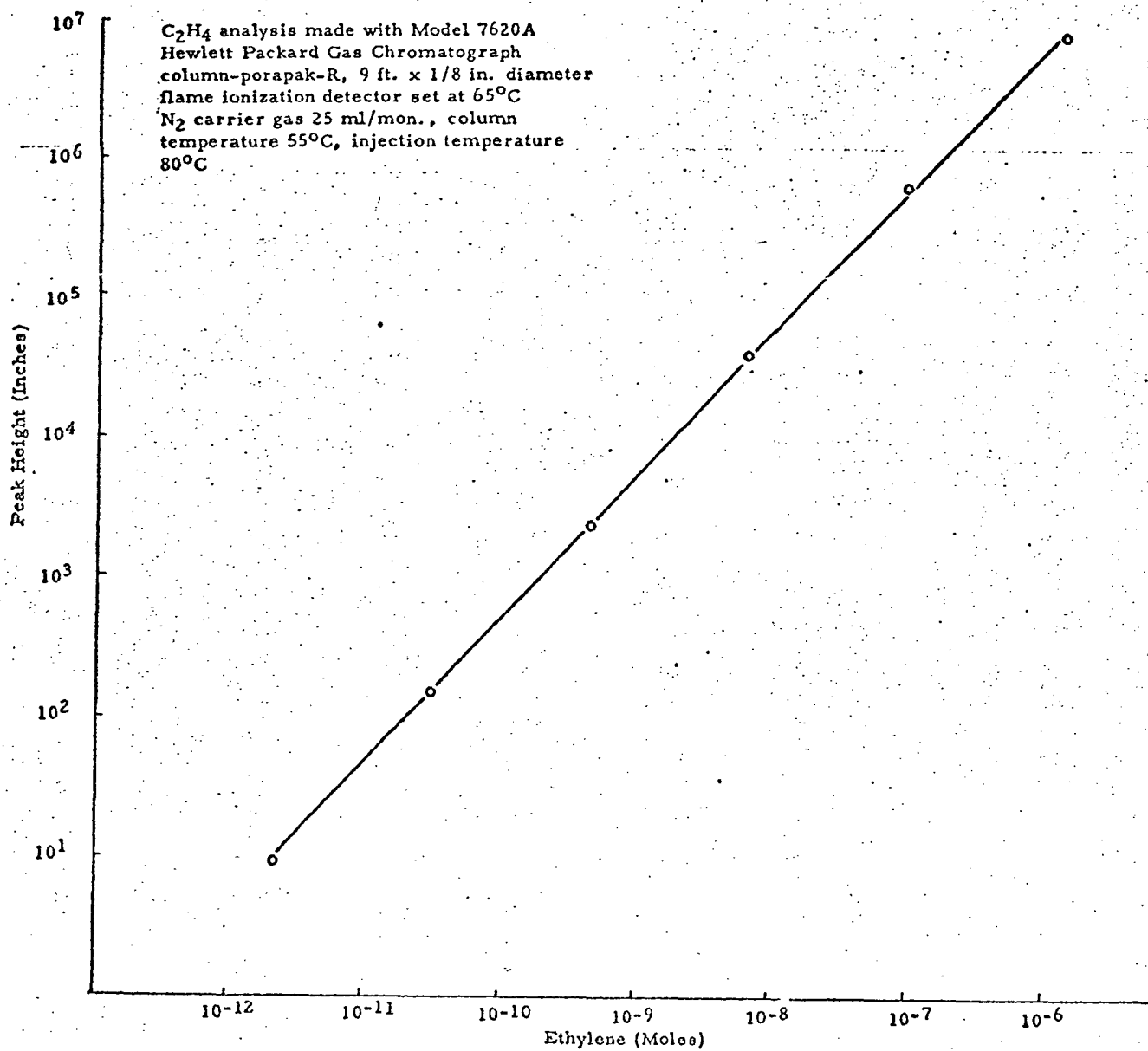
Ethylene Standard Curve Showing
Effect of Syringe Contamination
at Low C_2H_4 Concentrations



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FIGURE 52

Ethylene Standard Curve



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The experiment shown in Table 7, although preliminary in nature, pointed up the potential possibilities of the method as a life detection test. A test sample of Rock Creek mud gave a 10-fold production of ethylene as compared to an empty vial containing acetylene or samples of Wyaconda soil incubated under identical conditions. Also interest was the fact that 2 g samples of Wyaconda soil produced an average of three fold more ethylene in sunlight than under conditions of normal laboratory light. The contamination of ethylene in acetylene was found to be approximately that found in the negative controls. A more highly purified acetylene (Matheson) was obtained for future studies.

On the basis of the above results, a series of control experiments was conducted to determine the effect of light on biological and non-biological ethylene production. Glass and quartz incubation vials of approximately equal volume and configuration were used. Artificial illumination was provided by a light table containing 25 watt tungsten and power groove light bulbs (total illumination of approximately 400 foot candles. Temperature was maintained at approximately 26°C. No attempt was made to maintain temperature of the vials incubated in natural light. These vials were also subject to normal daily periodicity of light.

As shown in Tables 8 and 10, the vial type and source of illumination had no appreciable effect on nonbiological production of ethylene. The Gaithersburg field soil gave essentially the same response under all

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3. Results

TABLE 7

Detection of Nitrogen Fixation by Soil and Clover Root Nodules

<u>Experimental Conditions</u>	<u>Moles of Ethylene Produced</u>
(Negative Control)	
Empty Vial	
0.1 atm Acetylene in Flush Gas	1.3×10^{-9}
Incubation = 25°C, 96 hrs.	1.2×10^{-9}
Glass Vials, Fluorescent Room Light	1.2×10^{-9}
	Avg. 1.2×10^{-9}
(Positive Control)	
300 mg Clover Root Nodules	
0.1 atm Acetylene in Flush Gas	6.4×10^{-7}
Incubation = 25°C, 96 hrs.	5.6×10^{-7}
Glass Vials, Fluorescent Room Light	6.9×10^{-7}
	Avg. 6.3×10^{-7}
Approx. 1 g Wet Rock Creek Mud	
0.1 atm Acetylene in Flush Gas	1.6×10^{-8}
Incubation = 25°C, 96 hrs.	4.2×10^{-8}
Glass Vials, Natural Sunlight	Avg. 2.9×10^{-8}
2 g Wyaconda Soil (Fresh)	6.6×10^{-9}
0.1 atm Acetylene in Flush Gas	2.4×10^{-9}
Incubation = 25°C, 96 hrs.	9.7×10^{-9}
Glass Vials, Natural Sunlight	9.6×10^{-9}
	Avg. 7.1×10^{-9}
2 g Wyaconda Soil (Fresh)	
0.1 atm Acetylene in Flush Gas	1.2×10^{-9}
Incubation = 25°C, 96 hrs.	1.2×10^{-9}
Glass Vials, Fluorescent Room Light	Avg. 1.2×10^{-9}
0.05 g Wyaconda Soil (Fresh)	1.2×10^{-9}
2 ml RM9 without Fixed Nitrogen	1.4×10^{-9}
0.1 atm Acetylene in Flush Gas	1.3×10^{-9}
Incubation = 25°C, 96 hrs.	3.5×10^{-9}
Glass Vials, Fluorescent Room Light	Avg. 2.0×10^{-9}

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TABLE 8

Effect of Glass vs. Quartz Vials and Natural vs. Artificial Light
on Nonbiological Acetylene Reduction

Vial Type	-Moles C ₂ H ₄ /0.5 ml Sample		
	Dark	Natural Light	Artificial Light
Quartz	-----	2.8 x 10 ⁻¹¹	1.7 x 10 ⁻¹¹ 2.1 x 10 ⁻¹¹ 3.6 x 10 ⁻¹¹ Avg. 2.5 x 10 ⁻¹¹
Glass	3.0 x 10 ⁻¹¹ 7.5 x 10 ⁻¹¹ Avg. 5.2 x 10 ⁻¹¹	5.3 x 10 ⁻¹¹	4.5 x 10 ⁻¹¹

Experimental Conditions

No soil Added
0.1 atm Acetylene in Flush Gas
Incubation = 26°C, 69 hours.

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TABLE 9

Effect of Glass vs. Quartz Vials and Natural vs. Artificial Light
on Acetylene Reduction by Natural Rock Creek Mud

Vial Type	Moles C_2H_4 /0.5 ml Sample	
	Natural Light	Artificial Light
Quartz	2.9×10^{-8} * 7.2×10^{-10} Avg. 1.4×10^{-8}	5.3×10^{-9}
Glass	2.0×10^{-11} 7.0×10^{-12} Avg. 1.4×10^{-11}	9.8×10^{-9}

Experimental Conditions

1 g Rock Creek Mud
0.1 atm Acetylene in Flush Gas
Incubation = 26°C, 69 hrs.

*. The large difference between viable responses is thought to have resulted from the nonhomogeneous nature of the Rock Creek Mud. No attempt was made to homogenize the sample which had noticeable pockets of green growth.

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TABLE 10

Effect of Glass vs. Quartz Vials and Natural vs. Artificial Light
on Acetylene Reduction by Natural Field Soil

Vial Type	Moles C ₂ H ₄ /0.5 ml Sample	
	Natural Light	Artificial Light
Quartz	5.7 x 10 ⁻¹¹	4.9 x 10 ⁻¹¹
	2.9 x 10 ⁻¹¹	5.8 x 10 ⁻¹¹
	Avg. 4.3 x 10 ⁻¹¹	Avg. 5.4 x 10 ⁻¹¹
Glass	5.7 x 10 ⁻¹¹	5.0 x 10 ⁻¹¹
	4.4 x 10 ⁻¹¹	3.6 x 10 ⁻¹¹
	Avg. 5.0 x 10 ⁻¹¹	Avg. 4.7 x 10 ⁻¹¹

Experimental Conditions

1 g Field Soil (Gaithersburg)
0.1 atm Acetylene in Flush Gas
Incubation = 26°C, 69 hrs.

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incubation conditions, and that level was essentially the same as that obtained from incubation of acetylene in an empty vial.

Rock Creek mud (Table 9) did give a positive response. In natural sunlight, quartz vials showed a greater response than glass vials. However, the responses from the two vial types differed less under artificial light. Natural light appeared to produce more activity than artificial light when quartz vials were used; however, the quantitation and duplication of experiments exposed to natural light was difficult. The decision was made to use artificial illumination in quartz vials for further testing.

To test the response obtained from nonviable soils, samples were heated at 215°C for 30 minutes prior to testing by the acetylene reduction technique.

TABLE 11

Nitrogen Fixation by Rock Creek Mud

Moles C ₂ H ₄ /0.5 ml Sample			
Viable Soil		Nonviable Soil	
Dark	Light	Dark	Light
2.5 x 10 ⁻⁹	2.3 x 10 ⁻⁷	2.1 x 10 ⁻¹¹	1.4 x 10 ⁻¹¹
3.9 x 10 ⁻¹⁰	2.5 x 10 ⁻⁷	1.5 x 10 ⁻¹¹	2.1 x 10 ⁻¹¹
Avg. 1.5 x 10 ⁻⁹	Avg. 2.4 x 10 ⁻⁷	Avg. 1.8 x 10 ⁻¹¹	Avg. 1.8 x 10 ⁻¹¹

Experimental Conditions

1 g Rock Creek Mud
0.1 atm Acetylene in Flush Gas
Incubation = 26°C, 64 hrs.

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TABLE 12

Attempt to Detect Nitrogen Fixation in Wyaconda Soil

Moles C ₂ H ₄ /0.5 ml Sample			
Viable Soil		Nonviable Soil	
Dark	Light	Dark	Light
8.8 x 10 ⁻¹²	9.6 x 10 ⁻¹² 8.0 x 10 ⁻¹²	9.0 x 10 ⁻¹² 8.1 x 10 ⁻¹²	4.3 x 10 ⁻¹² 5.0 x 10 ⁻¹²

Experimental Conditions

1 g Wyaconda Soil
0.1 atm Acetylene in Flush Gas
Incubation = 26°C, 64 hrs.

Rock Creek mud (viable) again produced an excellent response.

The production of ethylene incubated in the dark was two orders of magnitude greater than the heat sterilized control. The production of ethylene by mud in the light was two orders of magnitude greater than that incubated in the dark. Therefore, the overall viable response from 1 g of Rock Creek mud (light incubation of viable vs. sterile control) was four orders of magnitude.

The Wyaconda soil sample showed no viable response. These results indicate that the population of nitrogen fixing aerobic microbes in this latter soil, if present at all, is below the limits of detection.

Also interesting in this experiment is the fact that heat treated Rock Creek mud showed approximately 2-3 fold more ethylene than the heat treated Wyaconda Soil. There are three possible explanations:

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1. The soil itself is capable of catalyzing acetylene reduction.
2. Heat treatment did not kill all the soil microorganisms.
3. Heat treatment killed all organisms but did not completely deactivate the nitrogenase.

Evidence provided by a later experiment supports the third explanation.

A time course experiment involving viable and heat sterilized cultures of Azotobacter showed an initial rate of N_2 ase activity that was comparable to the viable cultures. This activity was relatively short lived and reached a plateau much below the viable response plateau. The existence of heat stable nitrogenase enzyme would explain the fact Rock Creek mud gave the higher fixing biomass.

A number of freshly collected and laboratory stored soil samples were tested by the acetylene reduction method. The concentration of acetylene was reduced in these experiments, as shown in Tables 13A and 13b.

TABLE 13_a

Attempts to Detect Nitrogen Fixation in Laboratory Stored Soils

Sample	Moles C_2H_4 /0.5 ml Sample	
	Viable	Sterile
C_2H_2 alone	3.2×10^{-11} 1.6×10^{-10}	-----
1 g Creek Bed Soil	5.6×10^{-11} 6.6×10^{-11}	1.2×10^{-10} 7.8×10^{-11}
1 g Woody Soil	1.2×10^{-10} 3.8×10^{-11}	9.4×10^{-11} 8.3×10^{-11}
1 g Victoria Valley Soil	7.6×10^{-11} 7.0×10^{-11}	1.4×10^{-10} 8.9×10^{-10}
1 g Mojave Soil	5.5×10^{-11}	1.3×10^{-10} 8.9×10^{-11}

Experimental Conditions

0.04 atm C_2H_4 in Flush Gas
Incubation = 26°C, 7 days

N.B. All soils had been air dried and stored in the Biospherics soil collection at least one year prior to testing.

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TABLE 13b

Detection of Nitrogen Fixation in Fresh Soils

Test Sample	Moles C_2H_4 /0.5 ml Sample	
	Viable	Sterile
C_2H_2 alone	5.8×10^{-11} 6.1×10^{-11}	-----
1 g Wyaconda Mud (fresh)	9.5×10^{-10} 2.4×10^{-9}	7.6×10^{-11} 8.1×10^{-11}
1 g Rock Creek Mud (fresh)	1.6×10^{-9} 1.9×10^{-9}	7.0×10^{-11} 8.5×10^{-11}

Experimental Conditions

0.05 atm C_2H_2 in Flush Gas
Incubation = 26°C, 3.5 days

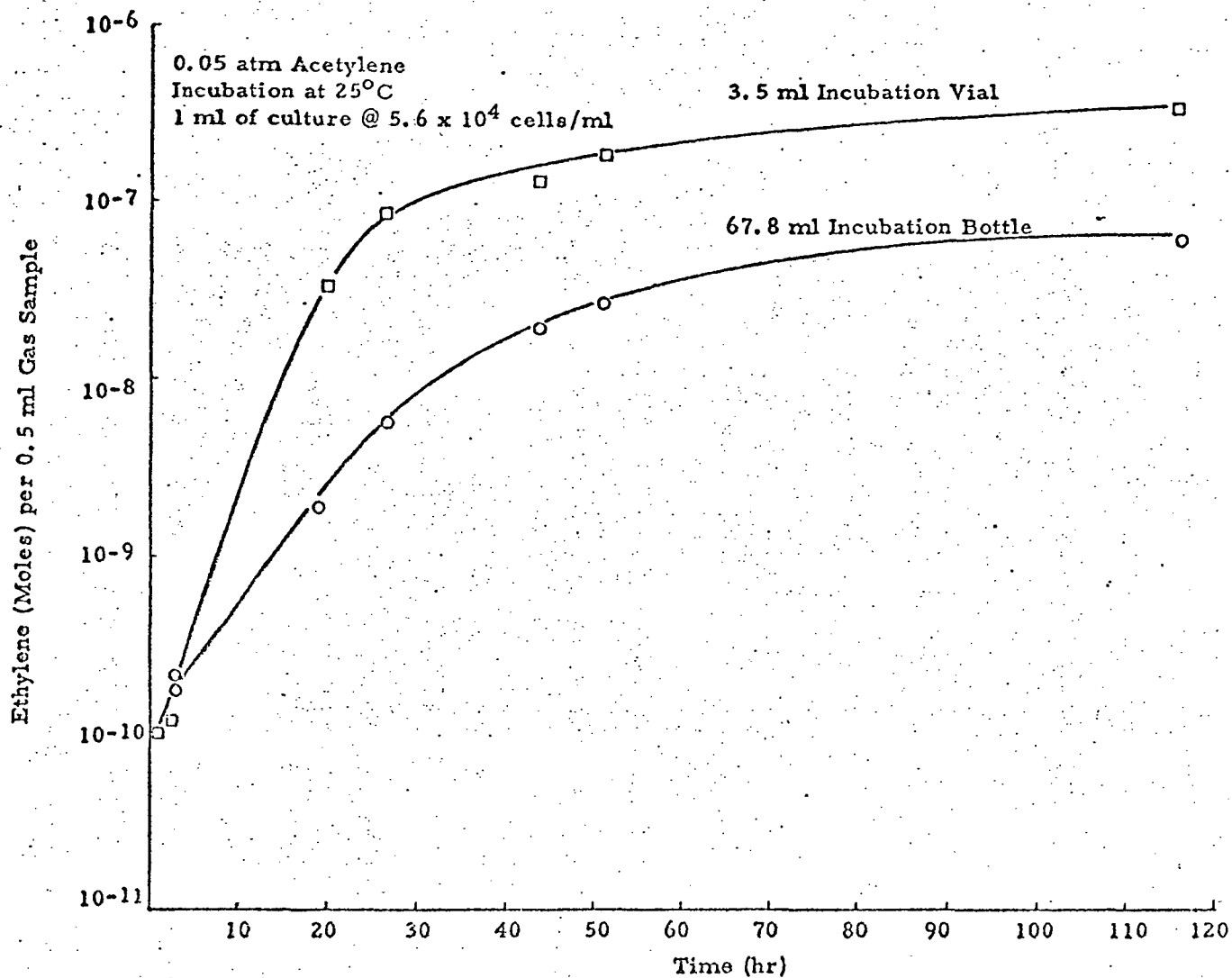
Nitrogen fixing activity was not demonstrated in soils which had been dried and stored for some time. The two freshly collected samples of mud from separate sources did show 20 to 30 fold increases in ethylene after incubation.

An experiment was performed to determine the time course of acetylene reduction from a pure culture of Azotobacter vinelandii. Two separate culturing systems were used. One involved 68.7 ml bottles in which 0.5 ml samples of gas were removed repeatedly from the sample bottle. The second experiment consisted of a series of 3.5 ml vials each of which was sampled one or two times. Figures 53 and 54 show the results.

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FIGURE 53

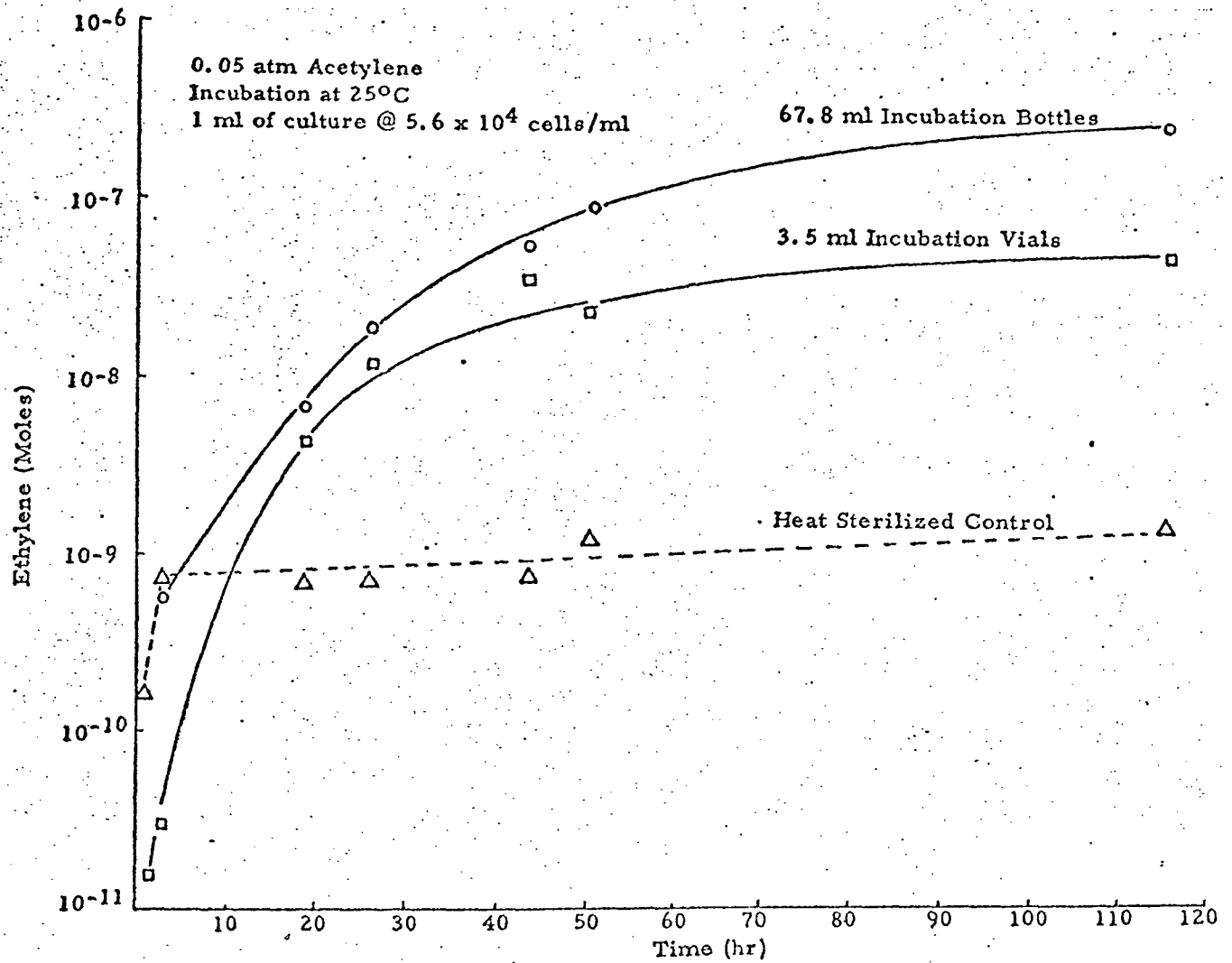
Acetylene Reduction by Azotobacter vinelandii



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FIGURE 54

Acetylene Reduction by Azotobacter vinelandii



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Maximum reduction of acetylene was achieved in both systems within approximately 50 hours. As shown in Figure 53, the measurable concentration of C_2H_4 was reached at a more rapid rate in the smaller vial. But a determination of actual C_2H_4 produced (Figure 54) showed that the initial rate of reduction was greater in the larger bottle and may have been a reflection of the larger surface area and shallow depth of sample which would allow a greater diffusion rate of acetylene to the cells.

The greater dilution factor resulting from the larger incubation vessel decreased the sensitivity of the method, but may have stimulated greater acetylene reduction. Both of these factors should be considered and tested in the design of an incubation chamber that would give maximum sensitivity.

The heat sterilized control (Figure 54) showed an initial increase in C_2H_2 reduction which stopped between one to three hours. The sterility of this sample was not tested; however, the failure of acetylene reduction to continue indicates that nitrogen fixing processes had stopped. It may be that the nitrogenase was heat stable and remained active until available ATP had been utilized.

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III. BIOPOLYMERS

A. Introduction

Development of a new non-geocentric life detection scheme based on the assumption that the presence of high molecular weight biopolymers (10,000) is indicative of live processes has been initiated. The rationale for this scheme is that biopolymers are a necessary constituent of terrestrial life required to code the complex and varied functions of life. Terrestrial examples include informational biopolymers such as nucleic acids, with a wide range of molecular weights up to several million, and proteins with an average molecular range from 10,000 to 500,000 (14). Even ferredoxin, which may be the most primitive protein known, ranges in molecular weight from 6,000 to 12,000 depending on the specific organism (15). Other high molecular weight biopolymers include the polysaccharides such as cellulose (mol. wt. range of 50,000 - 400,000), starch (mol. wt. range of 50,000 - several million), and glycogen (mol. wt. range of 270,000 - 100,000,000) and the complex lipids such as lipoproteins and lipopolysaccharides (14). While it is unlikely that Martian life would contain identical biopolymers, it is quite probable that extraterrestrial life would contain analogous complex informational biopolymers. It is the purpose of this life detection scheme to search for these biopolymers in soil. Since we know of no known natural processes whereby such high molecular weight polymers are produced nonbiologically, the detection of such material would be indicative of living systems. It is of

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note, however, that amino acids can abiotically condense on clay surfaces to form polypeptides ranging in molecular weight up to 2,000 and it has been suggested that similar processes may have played a catalytic part in the evolution of polypeptides (16). Further, high molecular weight "protenoids" have been artificially produced under simulated primitive reaction conditions although the evidence that similar processes occurred abiogenically in nature is inconclusive.

The search for biopolymers in soil assumes that if Martian life exists, it will exist in microbial form in soil. Thus, soil extraction coupled with grinding techniques designed to disrupt microbes should yield biopolymers along with a variety of smaller molecules which may or may not be of biological origin. It is recognized, however, that not all biopolymers are necessarily contained within microbes and some may be extracted from soil even without disrupting cellular membranes. For example, many terrestrial soil organisms produce exoenzymes such as nucleases, proteases, and phosphatases which aid digestive processes and which reside extracellularly in soil (18, 19). Once these biopolymers are obtained by extraction, the extract may be concentrated and passed through a molecular sieve column selected such that all material above a given molecular weight (for example, above 10,000) will pass directly through and be collected in a pre-selected fraction. Compounds of smaller molecular size will remain behind on the column. The isolated biopolymer fraction can be examined

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for presence or absence of specific high molecular weight components or groups of components.

The advantages of such a life detection scheme are numerous. Most important is its independence from specific metabolic pathways. Thus, it is a valid and reasonable test even if Viking '75 fails to provide a positive signal. Further, tests on the isolated polymers could also yield additional important information regarding biopolymeric structure(s) and possibly aid the design of future metabolic experiments. Other possible modifications include electrophoretic separation and examination for incorporation of a radioactively labeled precursor into the biopolymeric fraction thereby providing insight into Martian metabolism. All necessary operations are compatible with the AMML instrument concept.

One possible problem with this test is that the biopolymer concentration present in soil may be quite small and detection may require extremely sensitive techniques. For example, one report (18, 20) states that the amount of soil protein is very small relative to the amount of soil amino acids. In view of the many advantages, however, we have during this quarter examined the feasibility of detecting soil biopolymers. Our results indicate that the test is indeed feasible and that biopolymer fraction can be isolated from soil by molecular sieve chromatography. Details are presented in the following section.

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B. Experimental

1. Soil Extraction

For purposes of developing methodology for our new life detection scheme, we have examined protein as a model biopolymer and concentrated our efforts on its extraction from soil. Protein analyses have been determined using the Folin procedure of Lowry (21) and bovine serum albumin (BSA) as standard. This colorimetric analysis was chosen because of its ease of performance and its ability to detect as little as 10 μ g protein. The Folin test for protein depends upon the reaction of tyrosine and tryptophan with Folin reagent. The test is not absolutely specific for these aromatic acids since other low molecular weight compounds such as guanosine and uric acid and cysteine also react to produce color. However, by combining the test with preliminary trichloroacetic acid (TCA) precipitation, all acid-insoluble high molecular weight protein can be removed from all acid-soluble low molecular weight material, including free amino acids. Detection of Folin reacting material in the TCA precipitate is then indicative of protein. This combined procedure is simple, sensitive, and specific for protein material.

Our experiments were conducted with fresh Wyaconda (Rockville, Maryland) soil and were initially designed to optimize the extraction of all Folin reacting material from soil. In an initial experiment, we

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compared four procedures. In each of four separate vessels, 10 grams of soil were mixed with 10 ml of sterile distilled water. During the following 20 minutes, one portion was occasionally stirred, another was vigorously shaken, the third was ground with mortar and pestal, and the remaining portion was sonicated for three minutes. All procedures were conducted at 4°C. Following these operations, the extract was removed from the soil by centrifugation in a Sorvall refrigerated centrifuge at 3000 x g for five minutes followed by centrifugation at 7700 x g for five minutes. The results of this experiment are shown in Table 14. As shown, the extract obtained from sonication yielded the highest amount of Folin reacting material, indicating that such material can be extracted from soil. The other procedures (mixing, shaking, grinding) are not as effective but provide the control that the water used for the extraction procedures contributes little, if any, to the Folin reacting material extracted from soil by sonication.

Knowing the optimal sonication efficiency for this water extraction, we next attempted to improve the grinding procedure to match that of sonication. This was considered important because grinding is more adaptable to space instrumentation. The method used to improve grinding efficiency was reduction of the amount of solvent per gram of soil such that the resulting mixture formed an abrasive paste. After grinding this paste for 15 minutes at 4°C, additional liquid was added to increase the volume such that centrifugation would yield a liquid supernatant. Our experiments

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TABLE 14

Extraction of Folin-Reacting Material from Soil

Method of Extraction	Total μ g Extracted
Sonication (3 minutes), then Mixing	930
Grinding (Mortar and Pestal)	300
Vigorous Shaking	160
Mild Mixing	95

To each of four beakers was added 10 grams of fresh Wyaconda soil and 10 ml of sterile distilled water. During the following 15 minutes, the samples were treated as indicated. The supernatant was then clarified by centrifugation for 5 minutes at 3000 x g followed by centrifugation for 10 minutes at 7700 x g. All operations were performed at 4°C. Folin-reacting material present in the resulting extracts was determined by the method of Lowry (8) using BSA as standard.

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showed that this procedure successfully extracted approximately 75% of the total material obtained by sonication. Since the pH of the extract was 5.8, we compared sonication and grinding in Tris buffer (a mild chelating agent as well as a buffer). Utilization of 0.1 M Tris pH 7.0 improved the yield by perhaps 10-20% for both techniques although sonication was still superior to grinding. Thus, in one experiment comparing extraction of 20 g soil with 10 ml of 0.1 M Tris pH 7.0, sonication yielded 1.1 mg of Folin reacting material whereas grinding yielded 0.77 mg. Nonetheless, abrasive grinding was chosen as the standard technique and used for subsequent experiments. This technique had the additional advantage over sonication of better temperature control during extraction.

Having successfully extracted Folin-reacting material from soil, we next determined the percentage of this material attributable to high molecular weight protein. This was accomplished by precipitation of proteins with 7% TCA and subsequent removal of protein material from free amino acids and other low molecular weight compounds by centrifugation at 1,100 x g for 20 minutes at 4°C. The BSA standards used to quantitate the colorimetric assay were similarly treated with TCA. Our first experiment indicated that most of the Folin-reacting material extracted from soil was low molecular weight acid-soluble material. Thus, the extract obtained from 20 g of soil contained approximately 590 ug of Folin-reacting material whereas the pellet derived from TCA

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precipitation of this extract contained no more than 8 ug protein. this amount approaches the lower limit of detectability, it may be concluded that no more than 1% of the extracted material is proteinaceous. These results agree with those reported by McLaren and Peterson(18) who have shown a significant amount of amino acids present in soil. Proteins on the other hand, are "...almost nil" in quantity although activities of individual enzymes can be detected.

To obtain a better quantitative estimate of the amount of protein actually extractable by these techniques, we then conducted an experiment using 1000 g of fresh soil. The yield of TCA precipitable protein obtained from this experiment was approximately 200 ug or 0.2 ug/g soil. This clearly establishes that high molecular weight protein material can be extracted from soil. Of the total Folin-reacting material extracted from this soil (about 15 mg), however, only 1.3% of it was precipitable protein. Since our experiments with molecular sieve chromatography will require at least 1 or 2 mg of protein, it is consequently necessary either to increase the yield per gram soil. Further experiments have examined additional methods to increase the yield.

In considering ways to improve the yield, we noted considerable daily variability (\pm 50%) in the amount of material extracted. While at least part of this variation results from differences in grinding technique, other factors must also be operative. In an attempt to delineate this variability, we have compared extractions from fresh soil and from soil

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stored in-house for one day (both soils obtained from the same location). The results are shown in Table 15. As shown, the total Folin-reacting material extracted from each soil is similar (the 10% difference in amount extracted is within the range of observed experimental variation). However, the total precipitable protein is 5-fold higher from the fresh soil than from the stored soil. This may reflect hydrolysis of soil proteins and emphasizes the necessity of utilizing fresh soil daily.

Classical methods of extracting soil organic matter require the use of strong alkali (usually 0.5 N NaOH) which will usually dissolve more than half of the total organic matter (20). Such treatment has thus far been avoided in our investigations because of the possibility of hydrolysing biopolymers. However, a recent paper (22) has reported the extraction of 9.8 mg of an active enzyme preparation from 240 g of clay loam using 0.2 N NaOH as the extracting solvent. This yield (40 ug/g soil) is considerably greater than any so far obtained in our experiments. Consequently, we have performed a similar experiment whereby 200 g of fresh soil was extracted by grinding with 90 ml of 0.2 N NaOH. After separating the extract by centrifugation as previously described, the extract was immediately neutralized with HCl and examined for Folin-reacting material. It was found to contain a total of 3 mg of Folin-reacting material of which 250 ug were TCA precipitable protein. While the total Folin-reacting material is similar to our previous results, this protein yield (1.25 ug/g)

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TABLE 15

Extraction of TCA-Precipitable, Folin-Reacting Material
From Soil Under Various Conditions

Expt. #	Soil Sample	Solvent	Folin-Reacting Material	
			Total μ g Extracted	Total μ g Precipitated
1	Fresh	0.1 M Tris pH 7.0	1900	76
	Stored 1 Day	0.1 M Tris pH 7.0	2100	16
2	Fresh	0.2 N NaOH	1700	320
	Fresh	0.2 N NaOH +0.01 M EDTA	2400	369

To samples of 200 grams of Wyaconda soil was added 80 ml of the indicated extracting solvent. Each sample was extracted at 4°C by grinding with mortar and pestal for 15 minutes. After centrifugation, the 7700 x g supernatant was tested for Folin-reacting material (8). TCA was then added to a final concentration of 7% and the precipitated material removed by centrifugation and tested for Folin-reacting material.

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soil) shows considerable improvement (6-fold) over the average yield obtained with 0.1 M Tris buffer as the extracting solvent.

In an attempt to increase this yield still further, it was noted that organic soil constituents may be complexed with polyvalent metals (20). Consequently, extraction with 0.2 N NaOH was compared in the presence and absence of the chelator ethylenediamine tetraacetic acid (EDTA). The results (Table 15) showed that EDTA enhanced the yield of TCA precipitable material by approximately 10% and provided the highest yield so far obtained (2 ug protein/g soil). Thus, the solvent finally selected for optimal extraction of protein is 0.2 N NaOH + 0.01 M EDTA. The final extraction procedure is given in Figure 55.

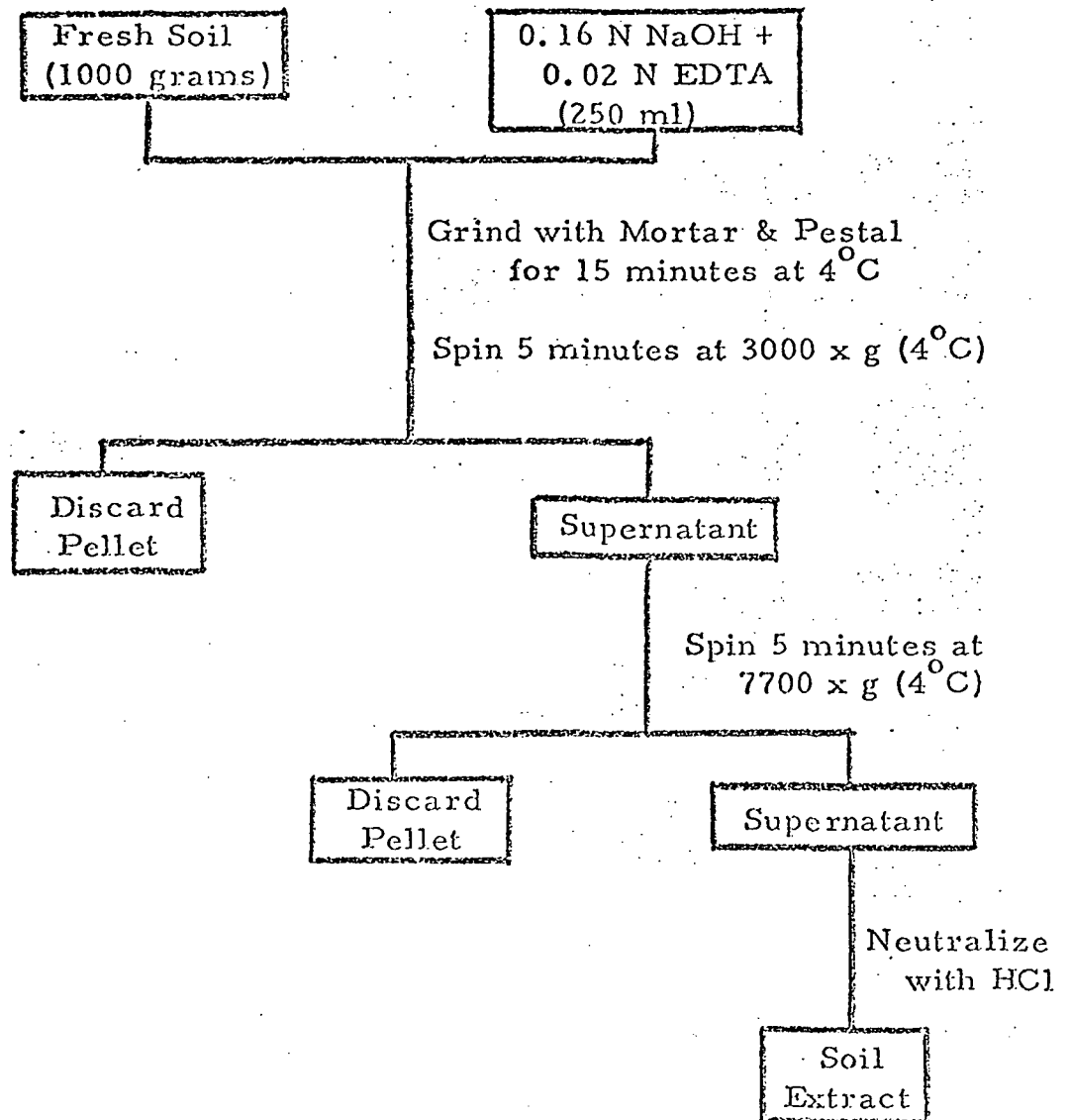
Since the extracting solvent must also remove other biopolymers from soil, a brief examination was made of the initial neutralized soil extract for polysaccharides which have been reported present in soil (23). Examination of the soil extract with anthrone reagent (24) gave a strong positive reaction indicated the successful removal of carbohydrate material from the soil. Experiments with molecular sieve chromatography (see later) indicated that a significant amount of this material was of high molecular weight.

It is concluded from these experiments that extraction of fresh soil with NaOH and EDTA successfully removes both protein and polysaccharide material. The following sections describe concentration of

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FIGURE 55

Soil Extraction Procedure



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these biopolymers and their subsequent isolation and detection via molecular sieve chromatography.

2. Concentration of the Soil Extract

Before applying the soil extract to a molecular sieve column, it is first necessary to concentrate it. This concentration can be accomplished either by specific precipitation of the extract constituents or by removal of water.

In considering specific precipitation methods, it has already been demonstrated that TCA can precipitate proteins from the extract. We have on several occasions also attempted to concentrate protein from the extract by precipitation with 80% saturated ammonium sulfate but these attempts were unsuccessful. Since these techniques are generally less desirable because they may fractionate biopolymers rather than concentrate them, further efforts to concentrate by specific precipitation have been abandoned.

Extract concentration by removal of water can be accomplished either by lyophilization or by flash evaporation. With flash evaporation, however, possible degradation may occur to biopolymers during the required mild heating. Consequently, we have selected lyophilization as the best method for concentration of our extracted biopolymers. One other possibility which we have not yet explored is concentration by the addition of solid Sephadex, a known method (25) for the concentration of high molecular weight material.

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3. Molecular Sieve Chromatography of Concentrated Soil Extract

Molecular sieve chromatography, or gel filtration, allows separation of molecules according to their molecular size (26). Two molecular sieve resins in common usage are Sephadex G (Pharmacia), consisting of a cross-linked polysaccharide dextran, and Bio-Gel P (CalBiochem), consisting of a cross-linked polyacrylamide. These resins exclude solutes of high molecular size, thereby allowing them to pass directly through the column, and retain smaller molecules which diffuse into the pores of the gel resin. Although it is generally assumed that elution patterns reflect molecular weight, it has been demonstrated (27, 28) that they actually show excellent correlation with the molecular radius or Stokes radius of the compound. A wide variety of these molecular sieve resins are available which are graded primarily according to their exclusion limit (lowest molecular weight compound that will pass directly through the resin bed without retention). For purposes of our experiments, we have arbitrarily selected 10,000 as a desirable exclusion limit and have consequently chosen Sephadex G-25 and Bio-Gel P-10 for study. Both resins are expected to exclude all compounds with molecular weights above 10,000 and to retain all molecules of smaller size.

In preparation of the molecular sieve columns to be used with concentrated soil extract, resin material of Sephadex G-25 and of Bio-Gel

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P-10 was equilibrated overnight in distilled water and then packed into a column with approximate bed dimensions of 1.5 x 20 cm. Each column was then calibrated by placing 0.3 ml of a mixture containing 2 O.D. units at 600 mu of blue dextran (Pharmacia; mol. wt. = 2,000,000) and 7 O.D. units at 259 mu of adenosine triphosphate (ATP; mol. wt. of disodium salt = 632). Flow rates were adjusted to approximately 30 ml/hour. Fractions were collected in 1.0 ml volumes with a Buchler Automatic Fraction Collector (Fractomat) and the optical density of each fraction determined in a Beckman DU spectrophotometer. Since blue dextran is completely excluded from each resin, its elution position indicates the elution position for all compounds with molecular weights above 10,000. This volume, known as the void volume (V_0), is characteristic of each resin and is dependent only upon the bed dimensions of the column. The determination of the elution volume (V_e) of ATP indicates the extent of separation of excluded compounds from those which are partially retained. In a second calibration run, a solution of 7 O.D. units at 600 mu of methylene blue (mol. wt. = 375) was also placed on each column to determine the elution position of a small molecular weight compound which would be completely retained.

Figure 56 shows the results of these calibration experiments for both Sephadex G-25 and Bio-Gel P-10. As shown, the exclusion limits (V_0) for these columns are 19 ml and 14 ml, respectively, and both

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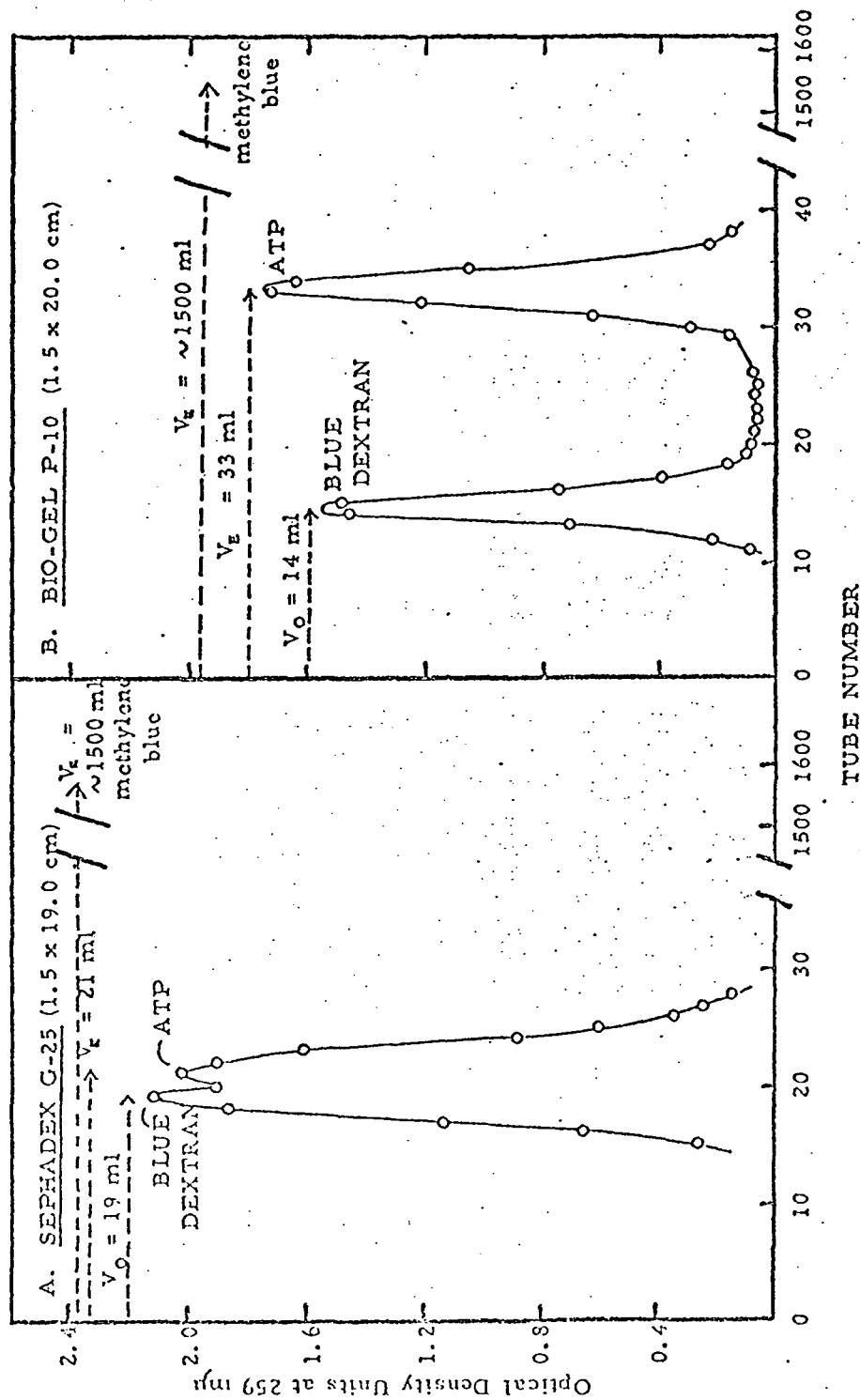


FIGURE 56

Molecular Sieve Chromatography of Blue Dextran and ATP. A 0.3 ml mixture of blue dextran (2 O.D. units at 600 mμ) and ATP (7 O.D. units at 259 mμ) was placed on columns containing Sephadex G-25 (A) and Bio-Gel P-10 equilibrated in distilled water. Fractions were collected in 1.0 ml volumes at an approximate flow rate of 30 ml/hr. The optical density of each fraction established the elution position of blue dextran (V_0) and of ATP (V_e). The elution position of methylene blue from each column was determined separately.

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columns retain methylene blue for approximately 1500 ml*. Thus, both columns readily discriminate high molecular weight material from smaller compounds (less than 400 molecular weight). Further, the high molecular weight fraction is collected within the first few milliliters eluted from the column. The elution volume for ATP on Bio-Gel P-10 is 33 ml which indicates excellent separation from high molecular weight polymers. However, ATP elutes from Sephadex G-25 at 21 ml and is separated from blue dextran by only 2 ml. Thus, because of better separation between high molecular weight material and compounds of intermediate molecular weight, Bio-Gel P-10 is better suited for our purposes.

The soil extract to be placed on each of these columns was obtained from 5000 g of fresh Wyaconda soil according to the procedure outlined in Figure 1. The final extract, which measured 340 ml, was separated into 75 ml portions and lyophilized over a three-day period. The lyophilized material originally from 75 ml of extract was then dissolved (or suspended) in 2.0 ml of distilled water and 0.5 ml of this mixture was placed on each column. This amount represents the extracted material from approximately 250 g of soil. The sample contained a considerable amount of brown material which remained near the top of each resin bed as elution proceeded. Fractions were collected in 1.0 ml volumes and

* Methylene blue may interact ionically with both columns since its retention time is considerably longer than anticipated. Further, methyl red (mol. wt. = 269) elutes from these columns at approximately 90 ml.

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the O.D. at 260 mu determined in a Beckman DU spectrophotometer.

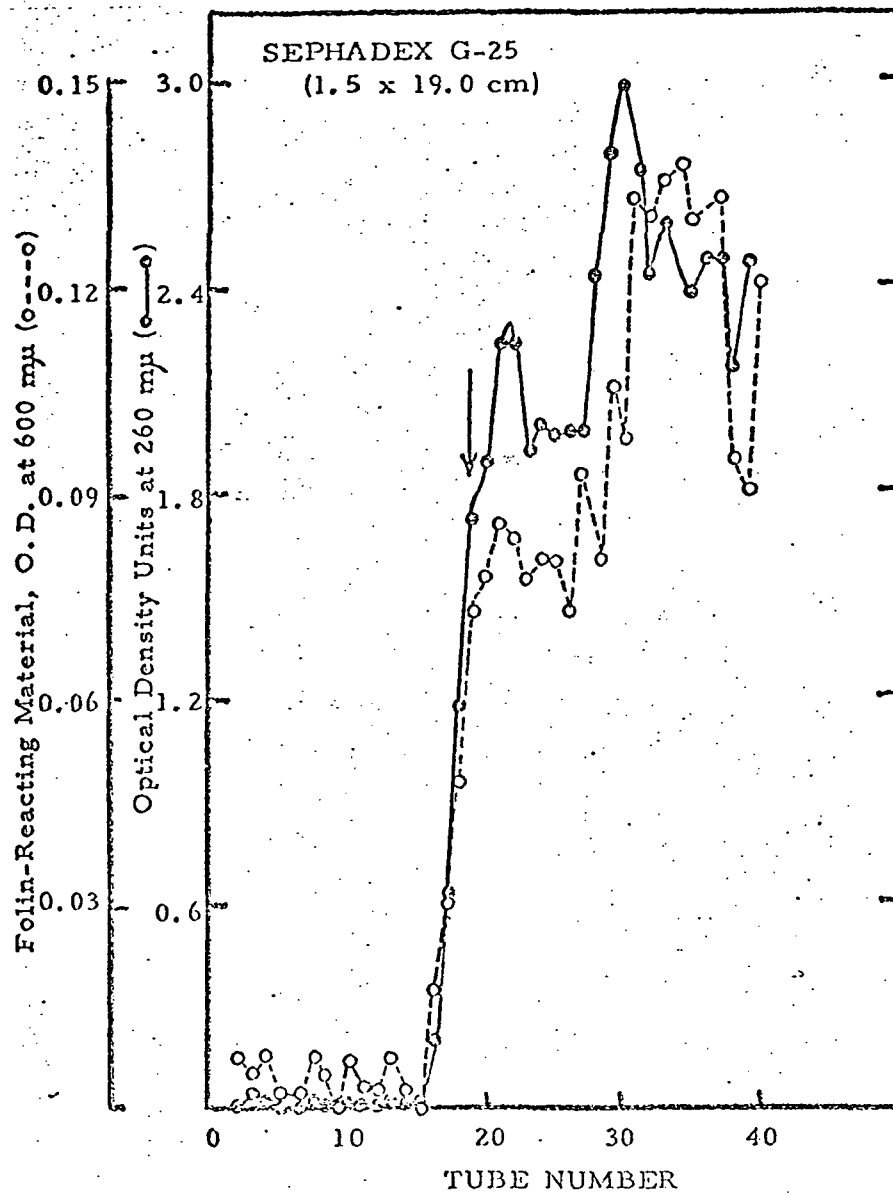
Following the optical density measurements, 0.5 ml was removed from each fraction and tested for protein content by the method of Lowry (21). With fractions from Bio-Gel P-10, consecutive fractions were next combined (i. e., tubes 1 and 2, 3 and 4, 5 and 6, etc) and 0.5 ml removed from each combination for determination of carbohydrate content by the anthrone reaction (24). Since the gel filtration had removed all low molecular weight material from these fractions, any positive reaction in the tube(s) representing the void volume is indicative of high molecular weight protein (Lowry) or polysaccharide (anthrone).

The results obtained from molecular sieve chromatography of concentrated soil extract are shown in Figure 57 and 58. The elution pattern as shown by optical density readings at 260 mu is indicated for each column. Both columns indicate that most of the eluted material is of low molecular weight, below 10,000. The elution pattern from the Sephadex G-25 column (Figure 4) shows a peak of material appearing at 21.5 ml, the approximate elution position of ATP (mol. wt. = 632). However, a leading shoulder also appears on this peak at 19 ml. Since this position is identical with that of the void volume for this column (Figure 2), the shoulder is indicative of high molecular weight material. That some high molecular weight material is indeed present is shown by the elution pattern from the Bio-Gel P-10 column. Here, a small but

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FIGURE 57

Molecular Sieve Chromatography of Concentrated Soil Extract

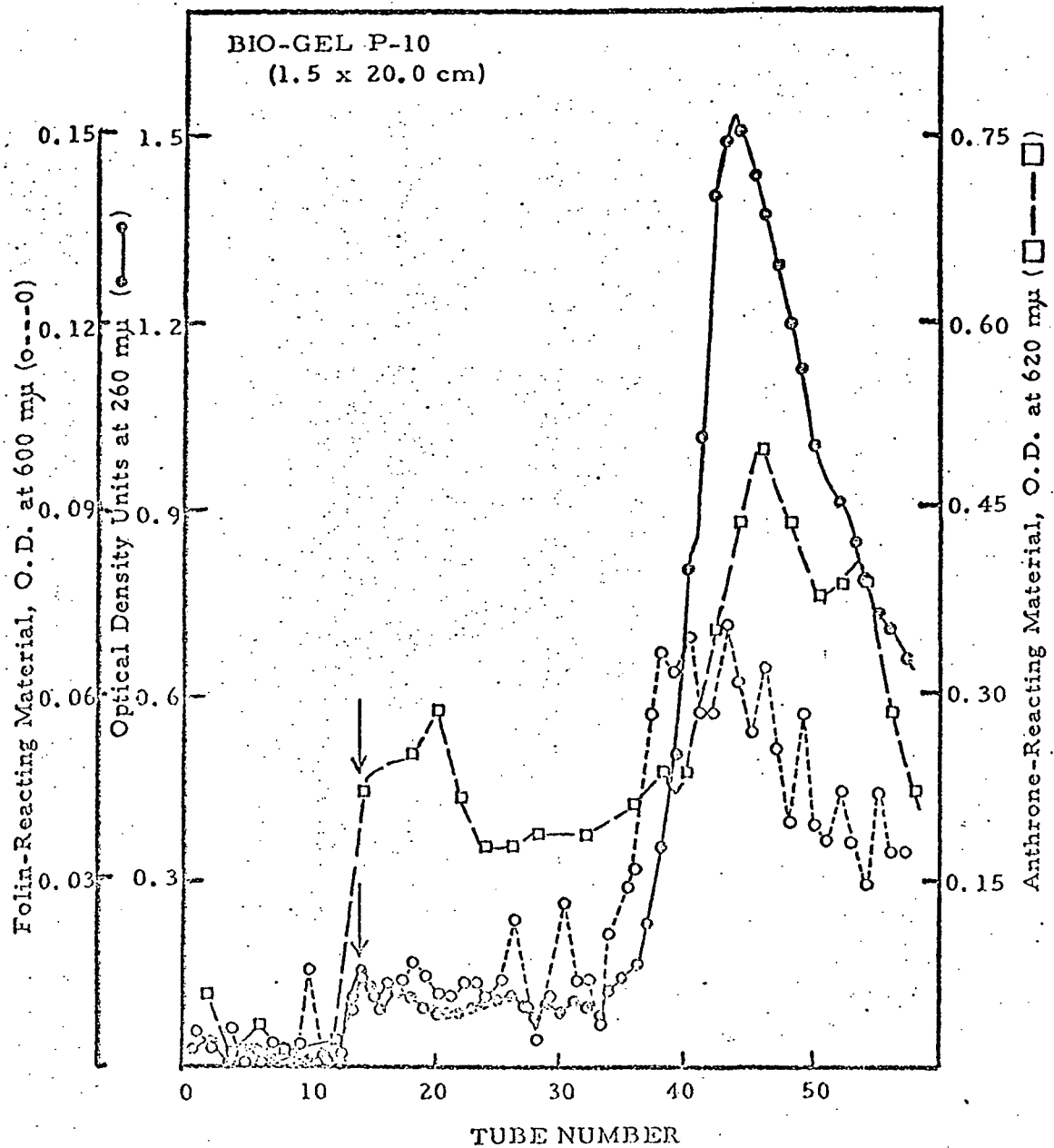


Soil extract from approximately 250 g of soil was lyophilized, concentrated into 0.5 ml, and placed on a column of Sephadex G-25 equilibrated in distilled water. Fractions were collected in 1.0 ml volumes and the optical density of each determined at 260 mμ (o-----o). Each fraction was then tested for the presence of Folin-reacting material (o-----o) by the method of Lowry (8). The position of the void volume is indicated by an arrow and was determined with blue dextran (Figure 2).

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FIGURE 58

Molecular Sieve Chromatography of Concentrated Soil Extract



Soil extract (0.5 ml) was placed on a column of Bio-Gel P-10 and fractions collected as described in "Figure 4". The optical density of each fraction was determined at 260 mμ (—○—). Each was then tested for Folin-reacting material (○---○) and for anthrone-reacting material (□---□). The position of the void volume is indicated by an arrow and was determined with blue dextran (Figure 2).

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distinct peak appears at the precise position established for the void volume of this column. Because of the degree of resolution between high molecular weight material and material of intermediate size, the Bio-Gel P-10 column thus provides evidence for the successful elution of polymeric material.

Figures 57 and 58 also indicate the amount of Folin-reacting material present in each fraction eluted from each column. Most of the Folin-reacting material is in the low molecular weight range, probably indicative of amino acids. However, Folin-reacting material also appears at the position of the void volume for both columns, indicating that at least some of the high molecular weight material is protein. It is of interest to note the size heterogeneity of Folin-reacting material eluted from Bio-Gel P-10 where material intermediate between 600 and 10,000 is present, possibly indicating peptides of varying lengths. That no fraction represents pure Folin-reacting material, however, is indicated by the fact that the 280/260 ratio throughout the elution profile remains approximately 0.890. Pure protein usually has a ratio of approximately 1.7 (21).

For the Bio-Gel P-10 column, the distribution of anthrone-reacting material was also determined and is indicated in Figure 58. As shown, most of this material is low in molecular weight, and, as anticipated, smaller than the average size of the low molecular weight Folin-reacting material. However, the sharp, pronounced shoulder appearing at the

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elution position of the void volume shows that some high molecular weight polysaccharide material is present.

It may be concluded from these experiments that high molecular weight material can be detected in concentrated soil extracts and separated by molecular sieve chromatography from low molecular weight material also present in soil. This high molecular weight material represents a small percentage of the total material present and consists of at least some protein and some polysaccharide material. These experiments thus demonstrate the feasibility of detecting biopolymers which are present in soil.

4. Stability of Sephadex G-25 and Bio-Gel P-10 to Heat Sterilization

Since utilization of the preceeding biopolymer experiment in planetary exploration would require sterilization of the molecular sieve resins, we have undertaken an examination of the heat stability of Sephadex G-25 and Bio-Gel P-10. That these resins are probably quite heat stable is suggested in a report whereby Sephadex G-25 and Bio-Gel P-300 were treated at 135°C for 36 hours (29). Although the resins were not examined per se, no changes in their properties were noted during these experiments. Further, Pharmacia claims that "...Sephadex may be sterilized in the wet state by autoclaving for 40 minutes at 110°C, without changes in properties or notable loss of material..." (30).

To test the heat stability of Sephadex G-25 and Bio-Gel P-10, samples of each resin were autoclaved both in the dry state and in the wet

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state for one hour at 120°C. When autoclaved wet, the gel was first equilibrated overnight in distilled water. After sterilization, the general appearance of the wet heat-treated resins was unchanged. However, when dry heat-treated, Bio-Gel P-10 formed a cohesive cake and Sephadex G-25 stuck somewhat to the flask. The resins were cooled overnight and those autoclaved in the dry state were then equilibrated in distilled water. Each heat-treated resin was next packed into a column 1.5 cm in diameter and approximately 20.0 cm in height for comparison to similar columns packed with resin material which had not been heat treated. Each column then received 0.3 ml of mixture of blue dextran (mol. wt. = 2,000,000) and methyl red (mol. wt. = 269). Methyl red is an indicator dye appearing yellow above pH 5.2 and red or pink at pH 4.2. Fractions were automatically collected and the optical densities determined at 600 mμ (blue dextran) and at 450 mμ (methyl red) to determine the elution pattern of each column.

Table 16 summarizes the results of this experiment. As shown, the elution or void volume (V_o) of blue dextran is not significantly affected by wet or dry heat treatment of either Sephadex G-25 or Bio-Gel P-10. Differences in the position of V_o for the most part reflect small differences in the volume of each column bed. However, the behavior of methyl red does appear to be affected by the heat treatment of the resins. Thus, when all six columns were first equilibrated in distilled water, methyl

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TABLE 16
Elution Behavior of Blue Dextran and Methyl Red Before and After
Heat Treatment of Sephadex G-25 and Bio-Gel P-10

Resin	Heat Treatment	Comment on Appearance After Treat	Columns in d. H ₂ O				Columns in Na acetate, pH 6.0			
			V _o (BD)	V _e (MR)	V _o /V _e	Color MR	V _o (BD)	V _e (MR)	V _o /V _e	Color MR
Sephadex G-25	None	—	24	39	1.6	Red	22	95	4.3	Red
	Wet Heat		25	32	1.3	Yellow	24	65	2.7	Red
	Dry Heat	Sticks to Flask	20	30	1.5	Yellow	24	90	3.7	Red
Bio-Gel P-10	None	—	16	45	2.8	Red	16	70	4.4	Red
	Wet Heat	—	17	21	1.2	Yellow	17	65	3.8	Red
	Dry Heat	Forms Cake	17	43	2.3	Red	16	70	4.4	Red

Samples of Sephadex G-25 and Bio-Gel P-10 were autoclaved for 1 hour at 125°C in both the dry and the wet (distilled water) state. Following cooling, each resin was packed into a column 1.5 cm in diameter and approximately 20 cm in height and compared to similar columns containing unheated resin. Columns were equilibrated either in distilled water or in sodium acetate buffer, pH 6.0, as indicated, prior to receiving 0.3 ml of a mixture of blue dextran (BD) and methyl red (MR). The elution position (in milliliters) of blue dextran (V_o) and methyl red (V_e) was determined by optical density measurements at 600 mμ and 450 mμ, respectively.

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red eluted faster (relative to blue dextran) from heat-treated resins than from the unheated resins. For both resins, this effect is most pronounced after wet heat treatment and is small after dry heat treatment. Further, although methyl red appears red in color on both untreated resins, it now appears yellow in three of the four heat-treated resins, indicating a pH change of the resin. The resin least affected in elution pattern is Bio-Gel P-10 heated in the dry state. This column also shows no change in pH as a result of heating.

In these experiments, it was noted that, whenever methyl red eluted faster after heat treatment, it also appeared yellow on the column. Consequently, in separate experiments, unheated resins were equilibrated at several pH values and methyl red alone placed on each. The results showed that at pH 7.5, methyl red appeared yellow in color and moved considerably faster than at low pH where methyl red appeared red. Thus, the color changed from red to yellow is apparently accompanied by an increase in molecular size (dimerization?). It may be concluded that, while heat treatment appears to affect the pH of certain resins, the resulting changes in elution pattern could reflect secondary pH effects rather than the separation properties per se of the resin.

To distinguish these possibilities, and also whether or not the changes caused by heating are reversible, each of the six columns was next equilibrated with several hundred milliliters of sodium acetate buffer,

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pH 6.0, and 0.3 ml of the mixture of blue dextran and methyl red placed on each. The resulting elution patterns (Table 16) confirm that the void volume is not significantly affected by heating. That the resin pH changes are reversible is indicated by the fact that methyl red now appears red on each column, indicating uniformity of pH. The elution patterns indicate little or no significant change in separation properties resulting from dry heat treatment of Bio-Gel P-10 or Sephadex G-25. However, both wet heat-treated resins show changed elution patterns. The change observed with Bio-Gel P-10 is relatively small whereas Sephadex G-25 shows large changes in separation properties as a result of wet heat treatment.

It may be concluded from these experiments that molecular sieve resins can undergo the heat sterilization prerequisite to planetary exploration. Thus, both Bio-Gel P-10 and Sephadex G-25 withstood dry heat treatment for one hour at 125°C without significant changes in separation properties. When heated in the wet state, however, both resins show small changes in separation properties. The resin of choice for dry heat treatment is Bio-Gel P-10.

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IV. MARS RETURN SAMPLE STRATEGY

A. Introduction

Although no manned missions to Mars are part of the current NASA project schedule, such missions will probably take place before the end of the century. Prior to effecting manned missions to Mars and the development of the necessary life support technology, NASA will undoubtedly have the capability to return samples of Martian soil to Earth by automated landers. From the standpoint of the health of future astronauts landing on Mars and of the public health in general following the return of such astronauts, a definitive examination of Mars for life prior to manned landings is essential. Any living forms present on Mars may prove to be hostile to terrestrial life. The same situation existed with respect to the Moon prior to the Apollo missions. Hence, extensive precautionary measures were taken upon the return of the first three astronaut crews and their spacecrafts. The possibility of life existing on Mars is considerably greater than was the case for the Moon prior to the Apollo missions. Hence, more elaborate precautions are warranted with respect to Mars. The initial approach to the development of a suitable strategy to cope with this problem is one of the objectives of the AMML program.

Upon recognition of the hazards that might accompany the return of astronauts or samples from other planets, the National Academy of Sciences

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in 1964 convened a conference on the potential hazards of back contamination from the planets. The lunar quarantine program was based on recommendations produced by the Conference. The same policy pertains to other planets. Some initial studies of the problem with respect to return samples from planets other than the Moon were conducted by the Jet Propulsion Laboratory as NASA Task No. 193-58-72-02 and were reported at the semi-annual NASA Planetary Quarantine Seminar held July 18 and 19, 1972 at San Francisco. At that meeting it was reported that the JPL effort specifically dedicated to this task had been terminated. However, further effort to define an acceptable level for the probability of Earth contamination will be continued under the Planetary Quarantine/Advanced Mission Task of JPL.

Biospherics plans to study this problem in more detail than heretofore in an attempt to devise a philosophy and a strategy which will be acceptable to the scientific community and to the public by way of assuring both groups that NASA can develop a workable and safe plan to permit Mars samples to be returned to Earth.

Much of the Planetary Quarantine Program centering upon means to prevent the contamination of Mars with terrestrial life has been devoted to determining probabilities of transport, survival, growth and dissemination of terrestrial life forms on Mars. While some probability and gaming may assist in the development of a program for the safe return of a Mars sample to Earth, it is recommended that the program be based on the assumption

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that the probability of indigenous life on Mars is one. Too many unknowns exist concerning the history of Mars, the nature of chemical and biological evolution and the adaptation of possible life forms to permit a realistic mathematical approach to the subject. Certainly, the introduction of any hazard to Earth must be viewed as a paramount concern. Hence, the program must be designed as if the object from which it seeks to protect the Earth actually exists.

B. Sample Acquisition

The following strategy is offered with respect to sample acquisition:

1. Representative Sampling

The samples should be representative of Mars. To accomplish this difficult task, return samples will be required from several landing sites which are determined to be typical of surface conditions and climates as determined by previous flybys, landers, orbiters, and by orbiters accompanying the landers designed to obtain return samples. This effort will constitute one of the most difficult portions of the planning in that no more than several return sample missions are probable because of the costs involved. Hence, careful examination of all available Mars data, life hypotheses, technological capabilities and limitations will have to be made by appropriate parties.

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2. Sampling Zone

The samples of Mars surface material should be obtained from beyond the zone of influence of the lander. This will require the development of sampler ejection devices which can be deployed from the landed spacecraft or from the spacecraft while in the Mars descent mode.

3. Sample Cleanliness

Sample acquisition must be performed in aseptic fashion and in a manner that does not add chemical contamination. These requirements stem from the possible influence of living or nonliving contaminants on the viability of any indigenous organisms and on the validity of the data to be obtained from biochemical, organic chemical and inorganic analyses of samples.

4. Sampling Strategy

Multiple, discrete samples should be obtained on conjunction with each landing mission. In the event the samplers are deployed from the landed spacecraft, an initial "blind" sample should be obtained immediately upon landing as a "contingency" sample. The subsequent samples would be obtained after suitable inspection and analysis of the area within range of the samplers. The specific sites should be selected in conformance with a pre-designed landing and sample acquisition strategy.

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5. In Situ Conditions

The acquisition and containment of the samples should be accomplished in a manner that will not disturb the major portion of the sample and, in effect, maintain in situ conditions.

6. Sample Containers

It is recommended that the samples be obtained and maintained as discrete, relatively small volumes. The sample containers should be designed such that, once sealed, they will preserve the native condition of the sample (perhaps supplying Martian gas headspace and providing a supply of Martian gas) for the duration of the return flight. In addition, the sample containers should be designed so that key analyses, including some for life detection, can be accomplished upon return of the sample without the necessity for breaking the sample container seal. Subsequent to such key analyses, the sample containers, maintained behind suitable biological barriers, should be opened under imposed Mars environmental conditions for completion of the total program analysis.

C. Sample Return

1. Acquisition of Sample Containers

The return of the samples must commence with the acquisition of the sample containers by the Earth return module of the spacecraft. In this module, the sample containers should be stored and maintained within the Mars ambient temperature range in the region from which they were

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taken. Artificial light in a diurnal cycle should be provided to at least one surface of the sample.

2. Intermediate Receiving Station

The return module of the spacecraft might be targeted to an intermediate receiving station. Preliminary examinations of the type most likely to produce evidence for life would be conducted at the intermediate station prior to returning the sample to a permanent station for complete analysis. The use of an intermediate station offers the advantage of obtaining important preliminary information which might influence the manner in which the sample is returned, handled or even whether or not it should be returned to Earth. Such an intermediate station might be established in the Moon as a part of experimental laboratories which will probably be established there within the next decade or two. An alternative intermediate receiving station could be aboard a laboratory spacecraft orbiting the Earth such as those presently planned for the Skylab Project. In either case, the initial analyses should be conducted under the supervision of trained biologists who would be in radio communication with a suitable planetary quarantine panel on Earth so that important information could be exchanged for timely decisions.

3. Permanent Receiving Station

Alternatively, the samples might be returned directly to a permanent station for analysis. The permanent station could be established

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on the Moon should our technology progress to that point prior to return Mars sample missions. It is more likely, however, that the permanent station would be established at a suitable location on Earth as was the Lunar Receiving Laboratory which served the Apollo missions. However, it is not believed that the present Lunar Receiving Laboratory can be readily adapted. Experience with the Lunar Receiving Laboratory indicate great difficulty in maintaining the biological barriers around the samples for the types of sample handling and analyses required. It is partly because of the difficulties experienced in the lunar programs that containment of the sample in its original container until key analyses have been performed is recommended. However, even this cautious approach will require means of assuring the prevention, removal or inactivation of any Mars surface particulates adhering to the external surface of the sample container as the result of its contact with or proximity to the Mars surface.

D. Sample Analyses

1. Objectives

Examination of the sample should have the following objectives:

- a. Detection of any type of life.
- b. Determination of infectivity of such life with respect to terrestrial life.
- c. Determination of predation of such life on terrestrial forms.

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- d. Determination of competition of such life with terrestrial forms.
- e. Determination of "environmental survival envelope" for any extraterrestrial organisms detected.
- f. Determination of control measures to produce stasis or tidal effects on any life detected.
- g. Determination of possible toxicity of Mars sample components for terrestrial life forms.
- h. Ancillary important information concerning the quality and quantity of life forms or toxic materials found.

2. Methods

The object of Table 17 is to present the broad outline of the types of information required and the proposed methods for obtaining the information in a manner compatible with the foregoing discussion. The methodology for conducting the analyses has been selected from the stand-points of suitability for automation, compatibility with conducting many of the key tests while the sample is contained in the specially designed sample containers, with emphasis on minimizing the number of different analytical techniques required and using, wherever possible, techniques already fully or partially developed by NASA technology. The table is intended to serve as an outline from which a detailed program can be developed. The latter must include designs for specific adaptation of the analytical techniques

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TABLE 17

Mars Return Sample Analysis

Determination	Method	
	Macroorganisms	Microorganisms
I. Morphology		
Shapes	Direct Imaging	Microscopy Imaging
Size Distribution	Direct Imaging	Microscopy Imaging
Motility	Direct Imaging	Microscopy Imaging with and without plating
Taxis	Direct Imaging	Microscopy Imaging
II. Abundance		
Horizontal	Direct Imaging	Microscopy Imaging of sample fractions: VLR, GEX, Plating
Vertical	Direct Imaging	Microscopy Imaging of sample fractions: VLR, GEX, Plating
III. Life Stages		
Forms	Direct Imaging	Microscopy Imaging
Life Span	Direct Imaging	Microscopy Imaging, plating, VLR, GEX
Generation Period	Direct Imaging	Microscopy Imaging, plating, VLR, GEX
Reproductive Methods	Direct Imaging	Microscopy Imaging, plating, VLR, GEX
IV. Metabolism		
Heterotrophy	Offer substrates: direct imaging, VLR, GEX, PRS	Offer substrates: VLR, GEX, PRS
Autotrophy	Expose to light; supply inorganic chemicals: VLR, GEX, PRS	Expose to light; supply inorganic chemicals: VLR, GEX, PRS
Nutrients	Offer nutrients: direct imaging	Offer nutrients: VLR, GEX, PRS
Temp. Response	Vary temperature: direct imaging, VLR, GEX, PRS	Vary temperature: Microscopy Imaging, VLR, GEX, PRS
pH Response	Vary pH: Direct Imaging, VLR, GEX, PRS	Vary pH: Microscopy Imaging, VLR, GEX, PRS
Water Response	Vary available water: Direct Imaging, VLR, GEX, PRS	Vary available water: Microscopy Imaging, VLR, GEX, PRS
Permeability	Vary substrates: VLR, GEX, PRS	Vary substrates: VLR, GEX, PRS
Diurnal Effects	Direct Imaging, VLR, GEX	Microscopy Imaging, VLR, GEX
Seasonal Effects	Direct Imaging, VLR, GEX	Microscopy Imaging, VLR, GEX
Composition and Metabolites	Before and after incubation: Pyrolysis, GCMS	Before and after incubation: Pyrolysis, GCMS
Inorganic	Pyrolysis, GCMS	Pyrolysis, GCMS
Organic	Amino Acid Analyses	Amino Acid Analyses
Amino Acids	Biopolymer (AMML)	Biopolymer (AMML)
Biopolymers	Direct Imaging, VLR, GEX, PRS	Microscopy Imaging, VLR, GEX, PRS
Atmospheric Response	ATP: Firefly bioluminescent assay	ATP: Firefly bioluminescent assay
Intermediary Metabolism		
V. Infectivity & Toxicity	Expose selected array of life forms: Direct Imaging	Expose selected array of life forms: VLR, GEX, PRS
VI. Predation and Parasitism	Expose selected array of life forms: Direct Imaging	Expose selected array of life forms: Microscopy Imaging
VII. Control		
Stasis and Cidal	Impose heavy metals, chemicals, organics, gases, heat, ionizing and nonionizing rad: Direct Imaging, VLR	Impose heavy metals, chemicals, organics, gases, heat, ionizing and nonionizing rad: Microscopy Imaging, VLR

GEX - Gas exchange
GCMS - Gas chromatograph - mass spectrometer
PRS - Pyrolytic Release

VLR - Viking labeled release
ATP - Adenosine triphosphate (firefly bioluminescent assay)
AMML - Automated Microbial Metabolism Laboratory

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and instrumentation of the return Earth module of the lander, sample acquisition system, sample container, biological barrier, analytical instrumentation, information analysis and decision making program.

Among the key inputs required for the expansion of Table 17 are the types and forms of terrestrial life to be exposed to the Mars samples to determine infectivity, toxicity, predation and parasitism. Considerable emphasis was placed on such tests in the Lunar Receiving Laboratory where a very wide variety of plants and animals were exposed to the lunar soil. In selecting terrestrial life forms to be exposed to Martian soil, it is suggested that the number of life forms be reduced to representative forms utilizing the different key biochemical pathways and cycles, and that more emphasis be placed upon microbial forms for bioassays. They offer the most direct method for exposing terrestrial life forms and can be readily monitored by a variety of rapid techniques, many of which have been developed under the NASA Extraterrestrial Life Detection Program.

E. Hazard Assessment

Upon completion of the examination of the Mars samples, a complete assessment of their hazards to terrestrial life must be made in order to decide whether or not the samples might be disseminated to other laboratories for further studies of a scientific nature. The results of the study would be directed toward determining the means by which such samples could be shipped, the nature of requirements to be imposed on the receiving

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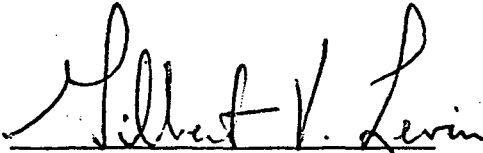
laboratory, the priority of types of experiments and any safety constraints to be imposed upon laboratory personnel.

Respectfully submitted,



J. Rudolph Schrot, Ph. D.
Research Microbiologist

Approved by:



Gilbert V. Levin, Ph. D.
Principal Investigator

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APPENDIX

TO

SECTION I. LABELED RELEASE

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MEDIA COMPOSITION

RM9

Basal: $\text{Na}_2\text{H PO}_4$ 5.0 mg/l
 $\text{MgCl}_2 \cdot 6 \text{ H}_2\text{O}$ 66 mg/l
 NH_4NO_3 0.20 g/l
 NaCl 0.10 g/l
 Soil Extract* 100 ml/l
 Tris 6.0 g/l
 pH 7.0

*Soil extract was prepared by suspending 500 g of air-dried Wyacoda soil in 1,300 ml of H_2O . The mixture was then autoclaved for one hour, filtered, and made up to one liter with sterile H_2O .

Radioisotopes: ^{14}C formate 1 $\mu\text{Ci/ml}$ $2.5 \times 10^{-4}\text{M}$
 UL^{14}C D-glucose 6 $\mu\text{Ci/ml}$ $2.5 \times 10^{-4}\text{M}$
 ULC DL-lactate 3 $\mu\text{Ci/ml}$ $2.5 \times 10^{-4}\text{M}$
 UL^{14}C glycine 2 $\mu\text{Ci/ml}$ $2.5 \times 10^{-4}\text{M}$

Modified RM9

Basal: Same as RM9

Radioisotopes: ^{14}C formate 1 $\mu\text{Ci/ml}$ $2.5 \times 10^{-4}\text{M}$
 UL^{14}C DL-lactate 6 $\mu\text{Ci/ml}$ $2.5 \times 10^{-4}\text{M}$
 UL^{14}C glycine 2 $\mu\text{Ci/ml}$ $2.5 \times 10^{-4}\text{M}$
 UL^{14}C L-alanine 3 $\mu\text{Ci/ml}$ $2.5 \times 10^{-4}\text{M}$
 UL^{14}C D-alanine 3 $\mu\text{Ci/ml}$ $2.5 \times 10^{-4}\text{M}$

VM1

Basal: Distilled H_2O

Radioisotopes: ^{14}C formate 2 $\mu\text{Ci/ml}$ $2.5 \times 10^{-4}\text{M}$
 UL^{14}C DL-lactate 12 $\mu\text{Ci/ml}$ $2.5 \times 10^{-4}\text{M}$
 UL^{14}C glycine 4 $\mu\text{Ci/ml}$ $2.5 \times 10^{-4}\text{M}$
 UL^{14}C L-alanine 6 $\mu\text{Ci/ml}$ $2.5 \times 10^{-4}\text{M}$
 UL^{14}C D-alanine 6 $\mu\text{Ci/ml}$ $2.5 \times 10^{-4}\text{M}$
 UL^{14}C glycolic acid 4 $\mu\text{Ci/ml}$ $2.5 \times 10^{-4}\text{M}$

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Preparation of RM9 Medium

- Step 1. Basal medium is made up in 1 liter batches, placed in 100 ml aliquots and autoclaved for 20 minutes, 15 psi, 121°C.
- Step 2. Labeled substrate is added to obtain the required radioactivity level. The concentration is then brought to the prescribed level with unlabeled substrate. After addition of labeled substrate(s) the solution is filter sterilized through a 0.22 u pore-size membrane filter.
- Step 3. Freshly prepared medium generally shows a higher than background count when the sterile medium is gettered. This non-biological emission can be reduced by shaking the sterile medium in a horizontal water bath shaker at 35°C. Medium is shaken until 0.2 ml shows a count of 100 cpm or less for a one-hour gettering period.
- Step 4. Radioactivity is measured before and after each experiment using the following procedure:
- Three drops of saturated $\text{Ba}(\text{OH})_2$ are placed in duplicate stainless steel concentric planchets.
 - To each of these planchets, 0.02 ml of medium and 0.04 ml of distilled water are added.
 - The planchets are dried under an infrared light for approximately 30 minutes.

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- d. Planchets are counted for one minute in a Nuclear Chicago D-47 Gas Flow Counting Apparatus.

Step 5. Media is stored at 3°C until used. It is filter sterilized again before use and measurement of radioactivity (as in Step 4) is performed prior to each experiment.

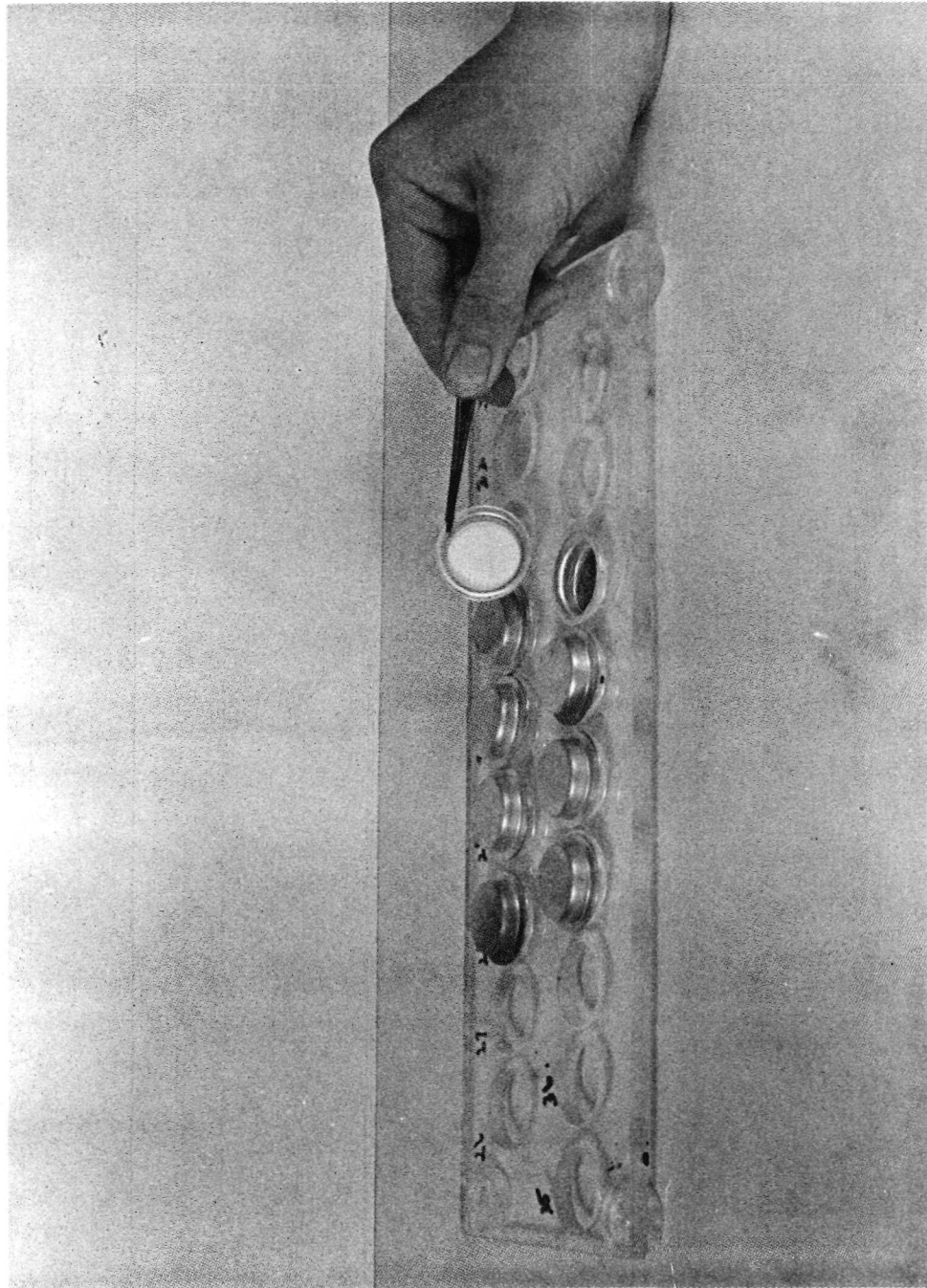
Procedure for Conducting the Labeled Release Experiment

- Step 1. Sifted soil samples (14 mesh screen), ranging in size from 25 mg to 100 mg, are weighed into sterile, one-inch aluminum planchets or adapted tubes (see Figures 1 and 2). Planchets are precleaned by boiling in Sparkleen detergent until water wets the aluminum evenly. They are then thoroughly rinsed with tap and distilled water.
- Step 2. Control soils (unless otherwise stated) are prepared by dry heat sterilization. Planchets containing a weighed quantity of soil are placed in glass petri dishes and placed in a hot air oven at 212°C for 30 minutes.
- Step 3. Planchets are placed in a holding rack or in sterile petri dishes and set on a laminar flow bench. An experiment is initiated by the addition of labeled medium to soil. Immediately upon addition of the labeled medium, each soil containing planchet is capped with an inverted planchet lined with a filter pad moistened with three drops of saturated $\text{Ba}(\text{OH})_2$.

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FIGURE 1

Culture Planchets Used for Conducting Labeled Release



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FIGURE 2
Culture Tubes Adapted for Conducting Labeled Release



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Step 4. After a timed interval, the gettering pads were replaced with planchets lined with freshly moistened $\text{Ba}(\text{OH})_2$ pads. This regimen was followed throughout the entire experiment.

Step 5. The getter pads were dried under an infrared lamp for about 15 to 20 minutes and transferred to clean planchets. Radioactivity retained on each pad was determined in a Nuclear Chicago D-47 Gas Flow Counting Apparatus flushed with Q-gas.